GAS CHROMATOGRAPHIC ANALYSIS OF INTERMEDIATES OF CARBOHYDRATE METABOLISM, AND ITS APPLICATION TO TISSUE.

A Thesis submitted for
Doctor of Philosophy Degree
in the University of London

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DEDICATION

This thesis is dedicated to the memory of my mother, Marjorie Joan Barnes, without whose efforts none of this would have been possible.

ABSTRACT

1. The dimethyl ester-trimethylsilyl ether derivatives of sugar phosphates were investigated and found not to be suitable for quantitative analysis.

2. The trimethylsilyl ester-trimethylsilyl ether (TMS) derivatives of sugar phosphates and other organic acids were prepared using the silyl amides, bis(trimethylsilyl)-acetamide and bis(trimethylsilyl)-trifluoroacetamide.

3. The silicone liquid phases SE-30, SE-52, OV-1 and OV-17 and the supports, Chromosorb W (acid washed, dimethylchlorosilane treated, 80-100 mesh) and Gas Chrom Q (100-120 mesh), were suitable for gas chromatographic analysis of TMS-derivatives.

4. Temperature programmed analysis from 100-240°C at 2° or 4°C per minute was used to separate the TMS-derivatives.

5. Quantitative data could be obtained for succinate, fumarate, malate, ∝-glycerophosphate, citrate, glucose, fructose-6-phosphate and glucose-6-phosphate when they were analysed as their TMS-derivatives by temperature programmed analysis.

6. The mass response curves of the phosphate containing derivatives did not pass through the origin in contradistinction to the non-phosphate containing derivatives.

7. The relationship between peak height and peak area was consistent, and either could be used in mass response curves.

8. Three methods of silylating tissue samples were investigated:-

Up to 10 mg of tissue which had been freeze dried could be silylated in 100 µl silylation reagent.

60% aqueous ethanol was the most suitable tissue extractant. Up to 100 mg of tissue which had been extracted with it could be silylated in 100 µl silylation reagent. A linear drop in sensitivity of detection was observed in these ethanolic extracts with increasing amounts of tissue.

Considerable purification of ethanolic extracts could be achieved using AG 1 (formate form) and AG 50(H^+ form) resins. Mass recovery figures indicated that there was a lower limit of 1g tissue which could be investigated.

9. The radioactive gas chromatography method has been used to study the effect of experimental diabetes on intermediate levels in rat heart, the effect of anoxia in rat heart on the metabolism of [U-^{14}C]-glucose and the differential metabolism of ^{14}C-glucose by the plasmodia of Physarum polycephalum.

10. In Appendix A.1 some observations on the carbohydrate biochemistry of P.polycephalum are noted.

11. In Appendix A.2 a computer programme for the calculation of peak areas in complex chromatograms is described.

DECLARATION

The experimental work in this thesis has been carried out by the author in the Department of Biochemistry, Imperial College between October 1967 and October 1970, and in the Department of Chemical Pathology, St. Thomas's Hospital Medical School between September 1971 and March 1972.

This thesis has not been presented in whole, or in part, for any other degree examination in London University, or elsewhere.

Signed ..... Date 12th September 1972.

Supervisor ..... Date 5th September '72

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ABBREVIATIONS

The author is using the abbreviations laid down by the Biochemical Journal in their instructions to Authors (1972) 126, 4-19. The following abbreviations apply to this thesis.

AW-DMCS	Acid washed, dimethylchlorosilane treated
BSA	N,O-bis-(trimethylsilyl)-acetamide
BSTFA	N,N-bis-(trimethylsilyl)-trifluoroacetamide
DPG	2,3-diphosphoglycerate
F6P	fructose-6-phosphate
FDP	fructose-1,6-diphosphate
FID	flame ionization detector
GLC	gas liquid chromatography
G1P	glucose-1-phosphate
G6P	glucose-6-phosphate
HMDS	1,1,1,2,2,2-hexamethyldisilazane
m/e	mass to charge ratio (in mass spectrometry)
PEP	phosphoenolpyruvate
2PGA	2-phosphoglycerate
3PGA	3-phosphoglycerate
6PG	6-phosphogluconate
PTFE	polytetrafluoroethylene
$Q_{O_2}^{air}$	metabolic quotient - μl O_2 consumed or evolved/mg tissue/hour
R_F	in TLC $\frac{\text{distance travelled by the compound}}{\text{distance travelled by the solvent front}}$
R_T	in GLC the retention time of a compound under certain defined conditions

TLC	thin layer chromatography
TMCS	trimethylchlorosilane
TMS	trimethylsilyl (of a derivative)
[U-^{14}C]	uniformally labelled with ^{14}Carbon
w/w	weight by weight
w/v	weight by volume

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CHAPTER 1

INTRODUCTION

1) Introduction

Previously quantitative estimation of the levels of carbohydrate intermediates in tissue have been made by enzymatic methods (see Bergmeyer, 1963). However, the specificity of the enzymes used meant that separate assays for each intermediate were necessary, consuming both time and sample. This approach can become too laborious unless only a few compounds are being studied. Other methods such as ion-exchange chromatography are even more laborious and although the many types of carbohydrate intermediates can be separated by this technique a single detection system for them all is not available, and thus at best multiple analyses are necessary. Therefore a method of estimation for the many intermediates of glucose metabolism in a single assay would be a large improvement on the current position.

Radioactively labelled materials have been used for the study of metabolic pathways, and in this the most useful measurement to be made was the specific radioactivity of each compound. To be able to estimate the specific radioactivity it is necessary to separate each compound first. The components of a pathway are generally closely related compounds and this can make their separation difficult. The bidemensional paper chromatographic method with automatic computer aided radioactive scanning described by Chain et al.(1971) achieved a separation of a wide range of compounds and determination of their individual radioactivities. However, mass measurements still had to be made separately for the determination of specific radioactivities.

With gas liquid chromatography (GLC) it is possible to determine the mass of a wide range of compounds in a single assay providing they are, or can be made to be by suitable chemical modifica-

tion, sufficiently volatile. It has been used with success in the study of fatty acid biosynthesis. It has the advantage over most other chromatographic techniques of being quantitative for the most part and also being amenable to simultaneous determination of radioactivities of the compounds being studied.

The purpose of this study has been to investigate and evaluate separation methods for the estimation of mass and radioactivity of the metabolites of glucose using radioactive gas liquid chromatography. It became necessary to devise extraction and purification procedures to clean up tissue samples so that the intermediates present could be studied.

Some applications of the analytical methods reported in this study have been applied to the metabolism of glucose in rat heart and in the plasmodia of the myxomycete, Physarum polycephalum. Since there was only a very scanty knowledge of the carbohydrate metabolism of the myxomycete some general studies of its carbohydrate metabolism were made, and these are reported in Appendix 1. As a rider to the analytical methods a computer programme for dealing with the chromatographic data was developed and its form is reported in Appendix 2.

2) Chromatographic separations of carbohydrate intermediates

Paper chromatography, thin layer chromatography (TLC) and ion-exchange column chromatography have been used for the separation of glycolytic intermediates. Both paper chromatography and thin layer chromatography suffer from the disadvantage of being qualitative rather than quantitative. It is possible to elute individual spots, but the elution is by no means quantitative for the levels found in

tissue samples (Kuksis & Prioreschi, 1967). These methods are however much simpler and cheaper than ion-exchange column chromatography or gas liquid chromatography.

Ion-exchange chromatography systems have been developed for the separation of sugar phosphates (Bartlett, 1959; Lefebvre et al, 1964). However, multiple detection systems are necessary for each type of compound being eluted. This involves collection of fractions and then time-consuming separate assays. The system described by Bedetti et al (1970) involved a 60 hour chromatographic separation, multiple concentration of the appropriate fractions to remove borate, and then enzymatic analysis.

In contrast gas chromatographic analyses rarely exceed two hours and the flame ionisation detector (FID) used in this study was both non-specific and linear in response over a wide range of concentration. Lack of specificity of detection may not be a serious disadvantage if resolution is adequate. Almost all organic compounds give a response to this detector.

3) Gas liquid chromatographic analysis of carbohydrate intermediates

GLC was initially developed for compounds which are naturally volatile at room temperature (James & Martin, 1952), or by suitable heating can be made to be volatile. It was extended to those compounds which cannot be made sufficiently volatile, even at high temperatures, by preparing volatile derivatives of them (James & Martin, 1956). Carbohydrates such as glucose cannot be chromatographed directly. The first volatile derivatives of carbohydrates to be prepared and assayed by GLC were the O-methyl ethers (McInnes et al, 1958).

A significant advance in the ease of preparation of a suitable derivative was made when the trimethylsilyl (TMS) ether derivatives of a very wide range of carbohydrates were prepared and chromatographed (Bentley et al, 1963). The method of preparation using a reagent containing anhydrous pyridine, hexamethyldisilazane (HMDS) and trimethylchlorosilane (TMCS) was very simple and was quantitative. The only disadvantage was that this method separated the individual anomers of each carbohydrate.

Attention was then given to the sugar phosphates and so glucose-6-phosphate (G6P), fructose-6-phosphate (F6P) and fructose-1, 6-diphosphate (FDP) were converted to volatile forms by first methylating the phosphate groups with diazomethane, and then forming the trimethylsilyl ethers of the sugar moeity (Wells et al, 1964). However, no further publications on this method or its application have appeared in the literature. The first section of Chapter 3 in this study is concerned with an examination of these derivatives.

A second derivative of the sugar phosphates has been proposed (Hashizume & Sasaki, 1966), the trimethylsilyl ester-trimethylsilyl ether. The use of this derivative for the examination of sugar phosphates after incubations with pure enzyme systems (Eisenberg, Jr. & Bolden, 1969; Sherman et al, 1969; Sherman et al, 1971) was reported during the course of these studies. However, no applications to compounds in tissue, or in extracts derived from tissue, have been reported. It was this derivative which was used for the determination of sugar phosphates in this study.

4) Gas liquid chromatographic analysis of compounds from the Krebs cycle

GLC analysis of the members of the Krebs cycle was first reported for their methyl esters (Kuksis & Vashwakarma, 1963). These derivatives have been used for the estimation of the acids in plant tissue (Kellog et al, 1964; Rumsey et al, 1964; Canvin, 1965). The problems associated with the much lower levels in animal tissue have been discussed by Kuksis & Prioreschi (1967) and Zaura & Metcoff (1969). A procedure for animal tissue using the recovery of added radioactively labelled compounds has been reported (Barnett et al, 1968).

An alternative derivative, the trimethylsilyl ester-trimethylsilyl ether, has been prepared for many of the Krebs cycle intermediates (Horii et al, 1965). The ∝-keto acids pyruvate, oxaloacetate, and ∝-ketoglutarate whether silylated or methylated give multiple forms because of enolisation. To prevent this Horii et al (1965) converted them to their oximes before forming the trimethylsilyl derivative. Similarly quinoxalones of ∝-keto acids were prepared by Hoffman & Killinger (1969). The trimethylsilyl ester-trimethylsilyl ether and dimethyl ester-trimethylsilyl ether derivatives of the Krebs cycle intermediates and other organic acids found in the urine have been studied by Dalgliesh et al (1966).

It therefore seemed possible by isolating the Krebs cycle intermediates with the sugar phosphates from tissue, to form trimethylsilyl derivatives of both groups of compounds and estimate all of them in a single chromatographic assay.

5) Radioactive gas liquid chromatographic analysis

The first apparatus to simultaneously measure the mass and radioactivity of a sample utilised a liquid scintillation cell placed after the mass detection system (a gas density balance) where the eluted materials from the column were condensed and counted (Popjak et al, 1959). Essentially the same apparatus was used in this study.

Although the scintillation cell measured the radioactivity less efficiently than the gas flow proportional counter (James & Piper, 1961, 1963), it was more sensitive overall since the whole peak was trapped in the cell. The flow through systems cannot contain all of each eluted peak inside the detector at once without losing the resolution obtained on the GLC column, and cannot retain the compound for long enough to measure the radioactivity present in weakly radioactive peaks. Other detection systems have used either a scintillation detector such as an anthracene crystal, the vapour passing over the crystal, i.e. a flow through system (Karmen et al, 1962b), or an ionisation chamber (Cacace et al, 1961).

The mass detector originally used for the liquid scintillation system was a gas density balance. With this detector there was no loss of sample during detection. The sensitivity of this detector was adequate for the concentration of fatty acids bound as lipid in tissue. However, in this study much greater sensitivity was required. The katharometer detector was even less sensitive than the gas density balance. Of the high sensitivity detectors the flame ionisation detector (FID) gave the most linear response. However, the sample was destroyed during the detection process. This could be overcome by the use of a stream splitter before the FID dividing the sample into two parts, one for mass detection, the other for radioactivity

detection. Splitters have been used for preparative chromatography (ratio 100:1) and with capillary columns (ratio up to 1000:1). A splitter was used with an apparatus with an FID mass detector and a gas flow proportional counter (Simpson, 1968). In this study a split ratio of 1:1 was investigated.

CHAPTER 2

MATERIALS AND METHODS

A. Materials

1) Chemicals and enzymes

The following materials were purchased from Boehringer (London) Corporation:

glucose-1-phosphate (disodium salt)

glucose-6-phosphate (disodium salt)

6-phosphogluconate (trisodium salt)

fructose-6-phosphate (disodium salt)

ribose-5-phosphate (barium salt)

fructose-1, 6-diphosphate (tetrasodium salt)

3-phosphoglycerate (trisodium salt)

2, 3-diphosphoglycerate (pentacyclohexylammonium salt)

α-glycerophosphate (disodium salt)

pyruvate (sodium salt)

succinate (disodium salt)

fumarate (disodium salt)

malic acid

oxaloacetic acid

α-ketoglutaric acid

L-malate : NAD oxidoreductase (E.C. 1.1.1.37)

citrate oxaloacetate-lyase (E.C. 4.1.3.6)

adenosine diphosphate

adenosine monophosphate

nicotinamide adenine dinucleotide

Peroxidase and Fermcozyme for the glucose oxidase assay were obtained from Hughes & Hughes Ltd. Ribulose-5-phosphate (barium salt) and 'Trizma' base were purchased from Sigma (London) Ltd. Absolute ethanol was purchased from Burroughs.

All other chemicals used were obtained from British Drug House Ltd. or Fisons, except where stated and were the best grades available.

2) Chromatographic materials

The ion-exchange resins AG 1-X4 (100-200 mesh) chloride form and AG 50W-X4 (200-400 mesh) hydrogen form were purchased from Biorad. Powdered cellulose CC41 was purchased from Whatman, and Kieselguhr G (nach Stahl) from Anderman.

Hexamethyldisilazane (HMDS), trimethylchlorosilane (TMCS) and N-methyl-N-nitroso-p-toluenesulphonamide were purchased from Kochlight. N,N,bis-(trimethylsilyl)-trifluoroacetamide and the silicone liquid phase SE-30 were purchased from Field Instrument Co. N,O bis-(trimethylsilyl)acetamide, the liquid phases SE-52 and OV-17, the solid support Chromosorb W (acid washed, dichloromethylsilane treated) 80-100 mesh, the column packings 3% w/w SE-30, SE-52 and OV-17 on Chromosorb W (acid washed, dichloromethylsilane treated) 80-100 mesh, and 0.25% w/w OV-17 on Corning glass beads 110 (80-100 mesh) and the 5' x $\frac{1}{4}$" outer diameter coiled glass columns were purchased from Phase Separations Ltd. The column packing 10% PEGA on Chromosorb G (80-100 mesh) and 5' x $\frac{1}{4}$" outer diameter coiled glass columns with a sealed glass to metal connection at the eluting end were purchased from Pye Unicam Ltd. The column packing 3% OV-1 on Gas Chrom Q (100-120 mesh) was purchased from Field Instrument Co. as was the methoxylamine hydrochloride.

Oxygen free nitrogen, compressed air and hydrogen were purchased from British Oxygen Co. and helium from Air Products Ltd.

All solvents were the best grades available and were redistilled in some cases. These were pyridine over potassium hydroxide pellets, acetonitrile over phosphorus pentoxide, and diethyl ether, ethyl acetate and triethylamine after drying first with anhydrous magnesium sulphate.

3) Chemicals for scintillation counting

2,5-diphenyloxazole (PPO) and 1,4-bis-2-(4-methyl-5-phenyloxazole)-benzene (POPOP) were obtained from Packard Instrument Co. Naphthalane (O.A.S. grade) was obtained from B.D.H. Toluene and 2-methoxyethanol were A.R. grades from Fisons Ltd.

4) Radioactively labelled chemicals

[U-^{14}C]-fructose, [1-^{14}C]-glucose, [2-^{14}C]-glucose, [6-^{14}C]-glucose and [U-^{14}C]-glucose, [U-^{14}C]-glucose-1-phosphate, [U-^{14}C]-glucose-6-phosphate, [U-^{14}C]-fructose-6-phosphate and [U-^{14}C]-fructose-1, 6-diphosphate, [1,5-$^{14}C_2$]-citric acid, [2,3-$^{14}C_2$]-fumaric acid, [2,3-$^{14}C_2$]-malic acid and [2,3-$^{14}C_2$]-succinic acid, [1-^{14}C]-n-hexadecane and [U-^{14}C]-Toluene were purchased from the Radiochemical Centre, Amersham.

B. Methods

1) The isolation of tissue and metabolic fixation

Method A

In metabolic experiments with myxomycete plasmodia, the time required for centrifugation was too long. To minimise metabolism after sampling, aliquots of the incubation mixture were pipetted

directly into liquid nitrogen in a beaker. The resulting frozen pellet was treated as if it were all tissue.

Method B

Since glycogen breakdown in rat liver was very rapid after death, the liver was freeze clamped *in situ* with tongs cooled in liquid nitrogen (Wollenberger et al, 1960). Rat heart was excised and washed with ice cold saline to remove blood before freeze clamping. Perfused rat heart was treated similarly to remove extracellular materials particularly glucose in the perfusion medium.

2) The extraction of frozen tissue with solvent

The frozen tissue was percussed in a stainless steel mortar cooled with liquid nitrogen, and then ground to a powder in a pestle and mortar cooled with solid CO_2. Cold (-20°C) 60% ethanol or in some cases 7.2% w/v perchloric acid was added to the powder (4 ml/g or 10 ml, whichever was the greater) and the mixture homogenised.

The homogenate was centrifuged (10,000 g for 10 minutes) in a refrigerated centrifuge at -5°C and the supernatant decanted. The pellet was further extracted with the same volume of 60% ethanol as before, the two supernatants being combined. A few drops of 0.880 ammonium hydroxide were added to make the extract slightly alkaline. The extract was concentrated to 1 ml under vacuum (water pump) using a rotary evaporator at 30°C. The extract was transferred to a centrifuge tube, and with washings of the flask made up to 5 ml. 5 ml magnesium acetate solution (30 mg) was added, followed by a few drops of 0.880 ammonium hydroxide. The white floc precipitate of magnesium ammonium phosphate was removed by centrifugation (2000 g, for 10 minutes) and the supernatant decanted carefully.

3) The preparation of ion-exchange resin columns

AG 1-X4 resin (100-200 mesh) chloride form was added as a slurry to fill a 12 x 0.5 cm column. Saturated sodium formate (50 ml) was passed through the column to convert the resin to the formate form. This was followed by formic acid (50 ml) and then with deionised distilled water until the eluant was almost neutral. The same procedure was carried out after the column had been used for the purification of a tissue extract. The flow rate through the column was increased to approximately 1 ml/minute by applying up to 5 p.s.i. of nitrogen to the reservoir.

AG 50W resin (200-400 mesh) hydrogen form sufficient to exchange all the sodium ions from 40 ml 0.5M sodium formate was added to a column 30 x 2.5 cms. The resin was washed twice with 40 ml 10M hydrochloric acid and then with deionised distilled water until neutral, usually three times with 40 ml was adequate. When regenerating, if ammonia had been used to elute the amino acids, the column was washed with water before passing through the 10M hydrochloric acid. Pressure was not needed to increase the flow rate with this column. Smaller 12 x 0.5 cms columns were prepared in the same way. Ground glass wool filters were used for the small columns, porous polythene disks for the large columns.

4) The purification of tissue extracts using ion-exchange resins

The extract was applied carefully to the AG 1 formate column. The column was washed with a further 9 x 10 ml deionised distilled water stepwise, each 10 ml being run through the column before the next 10 ml was added to the reservoir. This procedure removed all

the glucose (the precursor in incorporation studies). The carbohydrate monophosphates were eluted using 4 x 10 ml 2M formic acid, the diphosphates being retained completely. The myxomycete pigment was eluted as well to some extent.

The monophosphates and diphosphates were eluted with 4 x 10 ml 0.5M sodium formate. Sodium formate eluates were passed through the AG 50W (30 x 2.5 cms) column, a further 40 ml distilled water being added to wash the extract through. The formic acid solutions from the AG 1 columns were in earlier experiments extracted with 3 x one volume of ethyl acetate and then 3 x one volume of diethyl ether. This removed the pigment present. The samples eluted with sodium formate did not have contamination with pigment.

The formic acid solutions were finally evaporated to dryness under vacuum (water pump) using a rotary evaporator at 30°C. The dried residues were eluted with 3 x 1 ml 30% aqueous ethanol. Originally 80% aqueous ethanol was used, but losses of FDP were significant (see Chapter 5). The eluted extracts were pipetted into specimen tubes (75 x 12 mm). The aqueous ethanol was removed in a vacuum desiccator over phosphorus pentoxide overnight. A few µl of triethylamine were added when the tubes were almost dry (at the beginning of the day : by mid morning the tubes were dry).

5) The formation of dimethyl ester-trimethylsilyl ether derivatives of sugar phosphates

Method A

The method of Wells et al (1964) was used. Sodium salts of the sugar phosphates were passed through AG50W-X4 (20-50 mesh) hydrogen form resin (12 x 0.5 cm column) to convert them to the free acid. The

column was washed with 5 volumes of distilled water and the eluate concentrated to dryness under vacuum (water-pump) at 30°C using a Büchler Evapomix. Ethereal diazomethane was prepared by distilling the reaction mixture obtained by adding 40% KOH dropwise into an ether solution of N-methyl, N-nitrose-p-toluene sulphonamide containing a little methanol. A methanol solution (1 ml) of the sugar phosphoric acid was treated with ethereal diazomethane until there was a permanent yellow colour. The methylation reaction mixtures were taken to dryness under vacuum (water-pump) at 20°C, and were further dried by adding chloroform-methanol (4:1 v/v) and re-evaporating. The dry material was reacted with pyridine:HMDS:TMCS (10:2:1 by vol) at room temperature.

Method B

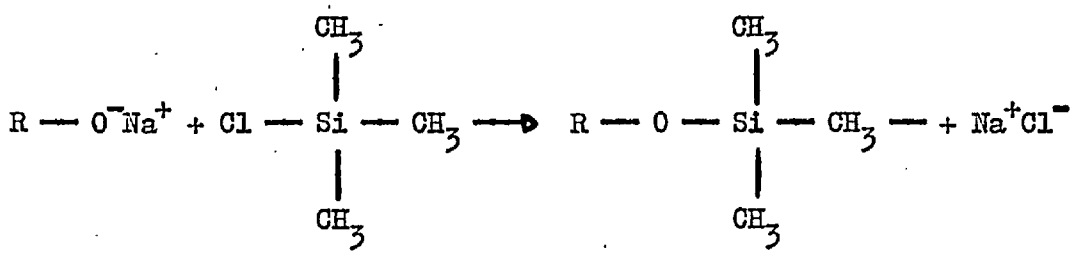
An alternative method of preparing the free acid of the sugar phosphates was to shake the sodium salt (10 mg) with AG50W-X4 (20-50 mesh) hydrogen form resin (1 g) in a test tube with 2 ml methanol. Vigorous shaking dissolved the salt. The solution was removed with a pasteur pipette taking care not to transfer any resin beads. The resin was shaken with another 1 ml methanol and this removed. The methanol solutions were combined and methylated with diazomethane and silylated as described above.

6) Formation of trimethylsilyl ester-trimethylsilyl ethers of carbohydrate intermediates

a) Reaction using the reagent of Bentley et al (1963)

This reagent consisted of anhydrous pyridine:hexamethyldisilazane (HMDS):trimethylchlorosilane (TMCS) (10:2:1, by vol). When mixed this reagent had a white opalescence which was cleared by

centrifugation (2,000 g for 5 minutes). Salts of the sugar phosphates and the Krebs cycle intermediates reacted slowly with this reagent (ratio 5 mg to 1 ml reagent), presumably because the TMCS reacted to form the acid



The ammonium salt reacted more quickly since the ammonium chloride formed was precipitated because of its insolubility in pyridine. Ammonium chloride was also formed by the combined actions of HMDS and TMCS on the hydroxyl groups. The free acids of intermediates (where stable) reacted most readily because of their greater initial solubility in the reagent.

The oximes and methoxime derivatives of the α-keto acids were prepared by reacting the salts or the free acids in pyridine (5 mg to 1 ml pyridine) containing 2 mg hydroxylamine or methoxylamine hydrochloride at 40°C for 45 minutes. Then 0.2 ml HMDS and 0.1 ml TMCS were added to form the trimethylsilyl derivative.

b) Reaction using bis(trimethylsilyl)-acetamide (BSA)

This compound could be used on its own to silylate the free acids, ammonium or triethylammonium salts of the intermediates (ratio 1-2 mg to 0.5 ml BSA). Each was freely soluble in the reagent at room temperature, or at 4°C. Auxiliary solvents were used with it, acetonitrile, pyridine and dimethylformamide. With TMCS present the sodium salts could also be reacted, an effect also reported by Sherman et al (1969). With material isolated from tissue a reagent consisting

of BSA, TMCS and pyridine (2:1:1, by vol) was used. Samples were stored overnight at 4°C to react.

c) Reaction using bis-(trimethylsilyl)-trifluoroacetamide (BSTFA)

This compound was used as for BSA, particularly for silylation of tissue extracts where 100 µl was used for the reaction. This reagent also contained added hydrocarbon standards, hexadecane and docosane (0.08 µmole of each per 100 µl).

7) Gas chromatographic analysis

a) The gas chromatography materials

All the silicone phases except OV-1 were coated onto Chromosorb W (acid washed dichlorodimethylsilane treated, 80-100 mesh) at a loading of 3% w/w. OV-1 was coated onto Gas Chrom Q (100-120 mesh) also at a loading of 3% w/w. Apart from the SE-30 column packing all the other column packings were purchased ready made.

3% SE-30 (methyl silicone) on Chromosorb W was prepared by dissolving SE-30 (1.5 g) in dichloromethane (200 ml), adding Chromosorb W (50 g) and removing the solvent under vacuum using a rotary evaporator in a 1 litre flask. The flask was rotated slowly, and evaporation was continued until the material was free running. The coated material was placed in a large petri dish and heated overnight at 100°C.

b) The loading of the column packing into the glass columns used for GLC

A silanised glass yarn plug was placed at the outlet end of the column (5' x ¼") and the glass to metal connector was attached (Pye Unicam). (NB - the rubber part of the connector was replaced by

one made of polytetrafluoroethylene (PTFE) so that analyses could be continued up to 250°C) For the recently introduced glass columns with the metal connector sealed into the glass the plug was not necessary as a porous sintered stainless steel plug was already present. The connector was attached to a vacuum line. The column packing was added in 1 g amounts with a filter funnel attached to the top of the column. Each aliquot was sucked through to the end of the column, the column being lightly tapped until there was no more settling before adding the next aliquot. The column packing was added until the level was 4.5 cms from the top of the column. Another glass yarn plug was pushed down on top of the packing to prevent the packing moving. The top 5 cms was replaced after every 50 injections.

c) <u>The treatment of the gas chromatographic column before analysis</u>

The column was placed in the gas chromatograph (Pye Unicam 104 series, models 44 and 64), without being connected to the detector to prevent contamination. Gas was passed through the column (20 ml/minute). For OV-17 helium was used, for the other liquid phases nitrogen was used. The temperature of the oven was raised to 50°C. Every two hours the temperature was raised a further 50°C until the maximum operating temperature was reached (for PEGA this was 200°C, for the silicone phases this was 250°C). The column was left at this temperature for 48 hours. The carrier gas was changed to nitrogen if helium had been used in the "bleeding in" stage, and the flow rate adjusted to 60 ml/minute at the temperature of operation. Measurement of flow rate were made with a soap bubble flow meter.

The column was connected to the inlet of the flame-ionisation detector and was left until the baseline was stable. For the column

packings, 3% SE-52 and 3% OV-17 on Chromosorb W (AW-DMCS, 80-100 mesh) it was necessary repeatedly (at least 12 times) to inject with BSTFA: pyridine:TMCS (2:1:1, by vol). A sample of TMS-G6P was injected and this repeated until consistent size peaks were produced. Each day before analyses were begun the column was injected with the silylation reagent and an aliquot of the first sample to be assayed at 250°C. At the end of each chromatographic run, an aliquot of the next sample was injected before cooling the oven. This was observed to give the most consistent results, presumably saturating all the active sites.

However, with 3% OV-1 on Gas Chrom Q (100-200 mesh) column packing, these methods were less necessary. The first injection onto a column containing this material produced a relatively normal chromatogram (see Figure 2.1); admittedly each intermediate was present at 25 nmoles, enough to saturate all the active sites. An important difference with this column packing was that the sample was injected into the head space above the column packing. This did not cause the severe discolouration which occurred when samples were injected into the column packing. It also increased the 'life' of the column and also meant that deactivation of the column each day was not so necessary. For example, the two chromatograms shown in Figure 2.2 were the result of injection of two identical aliquots of a standard solution, the first being injected only 40 minutes after starting up the GLC apparatus.

(contd. on p.38)

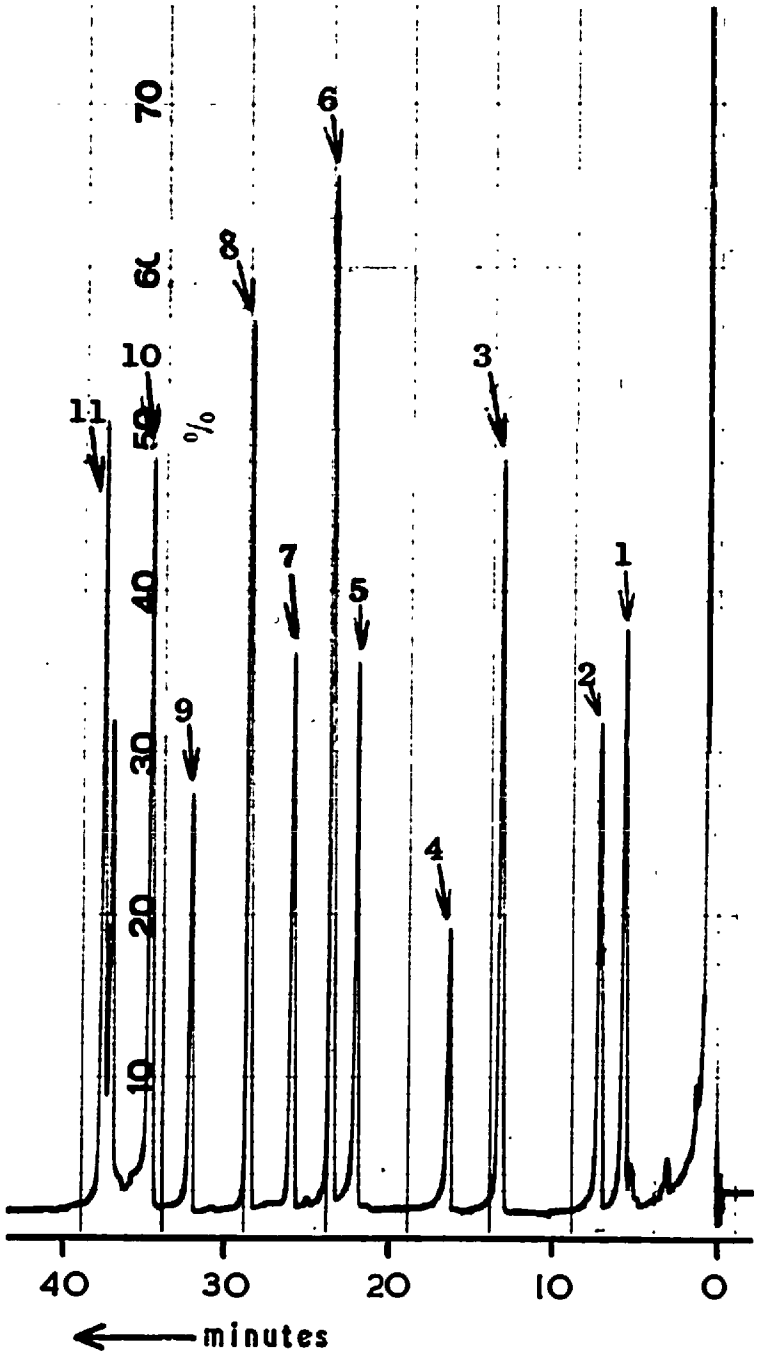

70
60
50
40
30
20
10
%
11
10
8
6
7
5
4
3
2
1
9
40
30
20
10
0
minutes

Figure 2.1 The gas chromatogram obtained for the first injection of TMS-derivatives onto a new 3% OV-1 on Gas Chrom Q (100-120 mesh) 5' x ¼" column

A 5 µl aliquot, containing 25 nmoles of each of the TMS-compounds, in BSTFA:pyridine:TMCS (2:1:1 by vol) was injected onto a new 3% OV-1 on Gas Chrom Q (100-120 mesh), 5' x ¼" column. The column after packing had been 'bled in' at 260°C. The temperature programme: 100°C isothermal for 5 minutes, then linear temperature programming at 4°C per minute 100-250°C was used.

Peak identification: 1. succinate 2. fumarate
3. malate 4. hexadecane 5. α-glycerophosphate
6. citrate 7. α-glucose 8. β-glucose
9. docosane 10. fructose-6-phosphate
11. glucose-6-phosphate

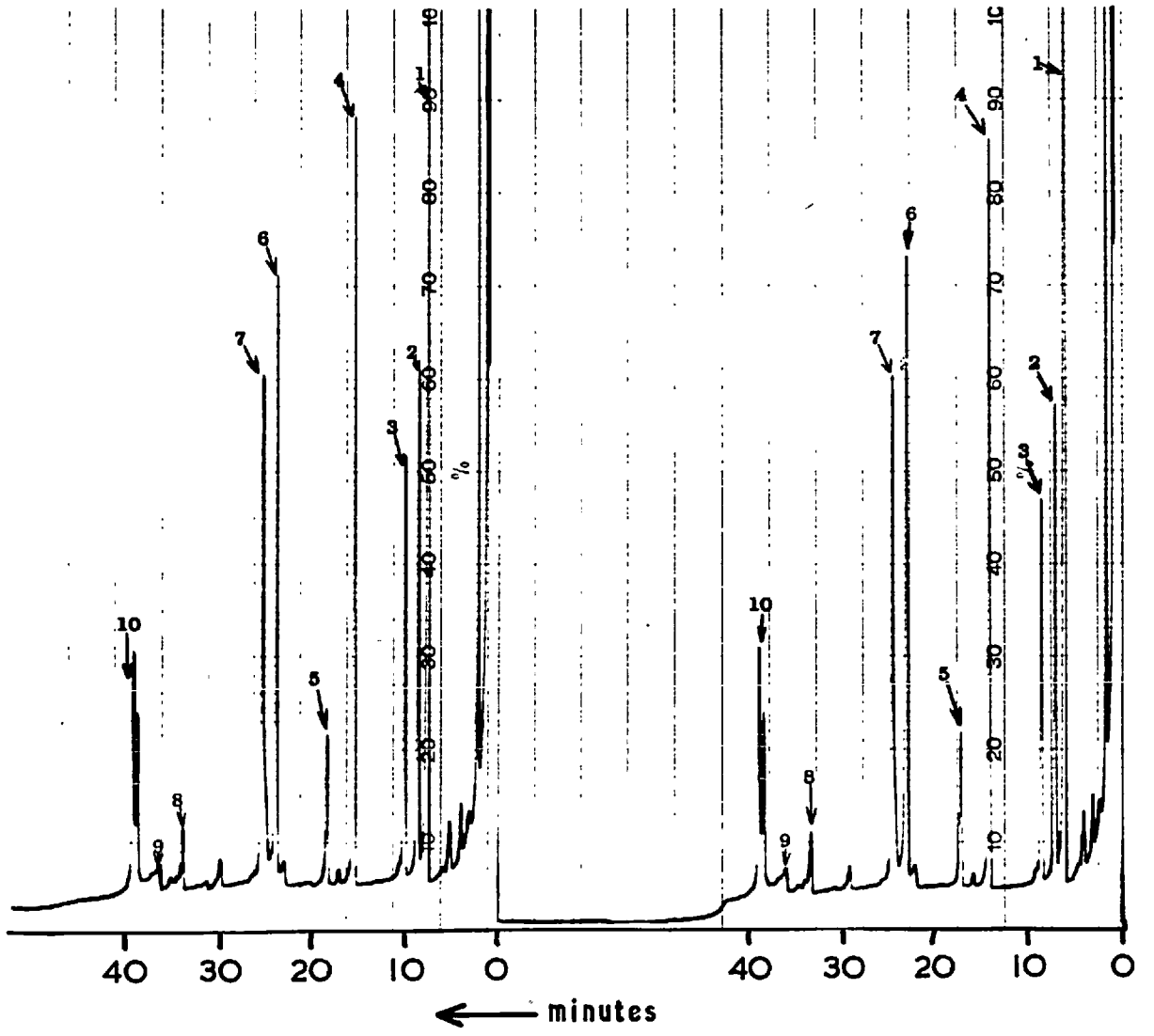

10
9
8
7
6
5
4
3
2
1
%
40
30
20
10
0
minutes

Figure 2.2 Comparison between successive injections of TMS compounds without prior column deactivation

Two 5 µl aliquots of a sample derived from tissue (a recovery experiment) were injected onto a 3% OV-1 on Gas Chrom Q (100-120 mesh), 5' x ¼" column. The GLC apparatus was started up, i.e. nitrogen gas flow, heating to 250°C and flame ignition, 40 minutes before the injection of the first sample. The temperature programme: 100°C isothermal for 5 minutes, then linear temperature programming at 4°C per minute 100-250°C, was used.

Peak identification: 1. phosphate 2. succinate
3. fumarate 4. malate 5. hexadecane
6. ∝-glycerophosphate 7. citrate 8. hexadecane
9. fructose-6-phosphate 10. glucose-6-phosphate

It is likely that injection onto the column packing caused stripping of the liquid phase from this part of the packing, thus exposing the support material and the active sites. Injection above the column packing resulted in the solvent being in the vapour phase before it reached the column packing and thus reducing the stripping out of the liquid phase.

8) The radioactive gas liquid chromatography apparatus

a) The scintillation cell

This was a modification of that described by Popjak et al (1959). Replacable transparent Melinex windows formed the walls of the cells unlike the original model where the faces of the photo-multiplier tubes were the walls of the cell. With other minor modifications the apparatus could be readily taken apart for inspection and then fitted together again. A fuller description of the modification is to be published (A.E. Lowe, personal communication).

The scintillation cell was emptied and filled by means of an automatic dispenser similar to that described by Popjak et al (1959). An important difference was that incoming fluid was loaded from the top, through the chimney, the cell being emptied from the bottom. This prevented fresh scintillant from being contaminated before entering the cell (see Figures 2.3 and 2.4).

The scintillation fluid consisted of 2,5-diphenyloxazole (PPO) 4g, 1,4-bis-2-(4-methyl-5-phenyloxazole)-benzene (POPOP) 0.2 g per litre of toluene. The scintillant (2.5 litres) was stored over 250 g anhydrous sodium sulphate to keep it dry. 'Wet' scintillant, or with an alcohol added, caused loss of radioactivity as shown in

(contd. on p.45)

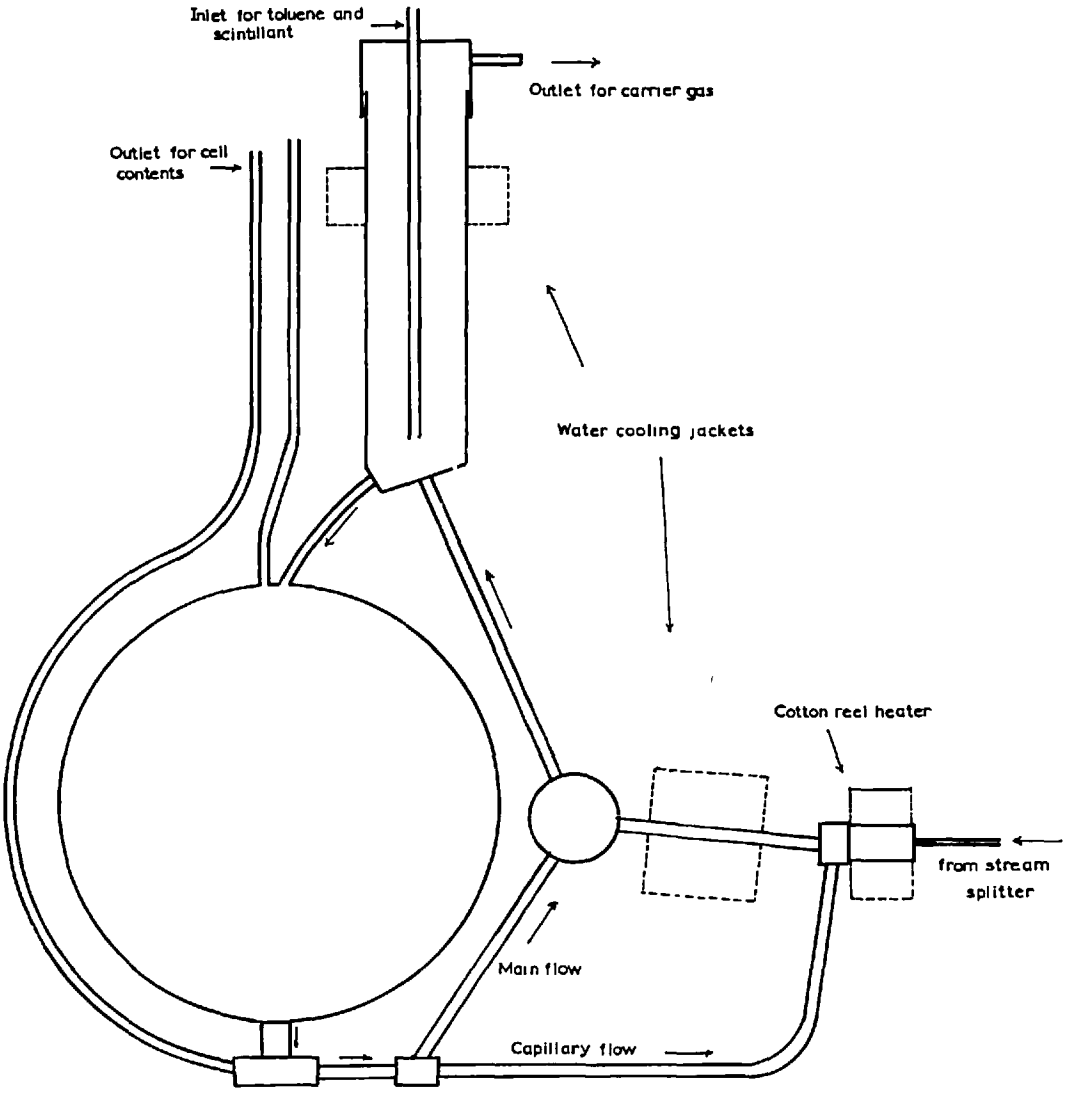

Inlet for toluene and scintillant
Outlet for carrier gas
Outlet for cell contents
Water cooling jackets
Cotton reel heater
from stream splitter
Main flow
Capillary flow

Figure 2.3 The scintillation cell for radioactive gas chromatography

This apparatus was similar to that reported by Popjak et al (1959). The arrows parallel to the pipes indicate the movement of the scintillation fluid.

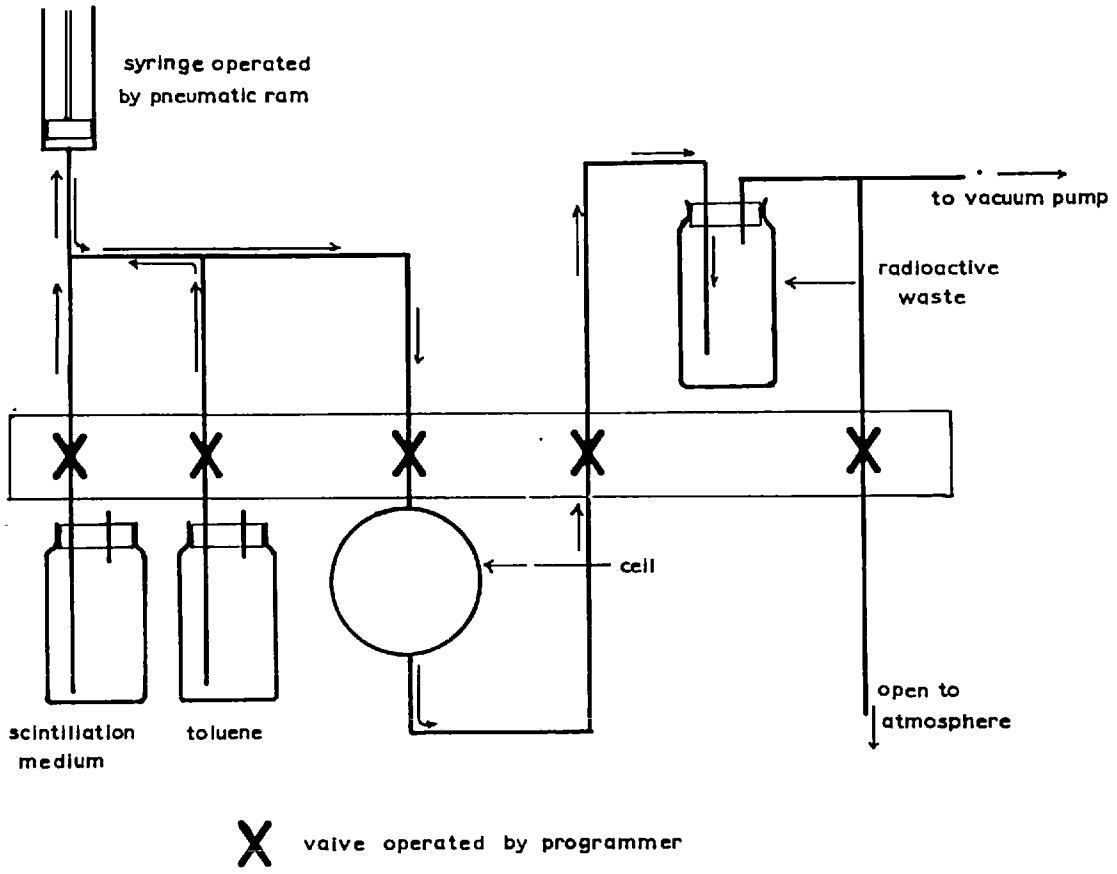

syringe operated
by pneumatic ram
to vacuum pump
radioactive
waste
cell
open to
atmosphere
scintillation
medium
toluene
valve operated by programmer

Figure 2.4 The automatic scintillant dispenser

This is a diagramatic representation of the apparatus. It was similar in principle to that described by Popjak et al (1959). The valves were controlled by a 6-channel Letromec programmer.

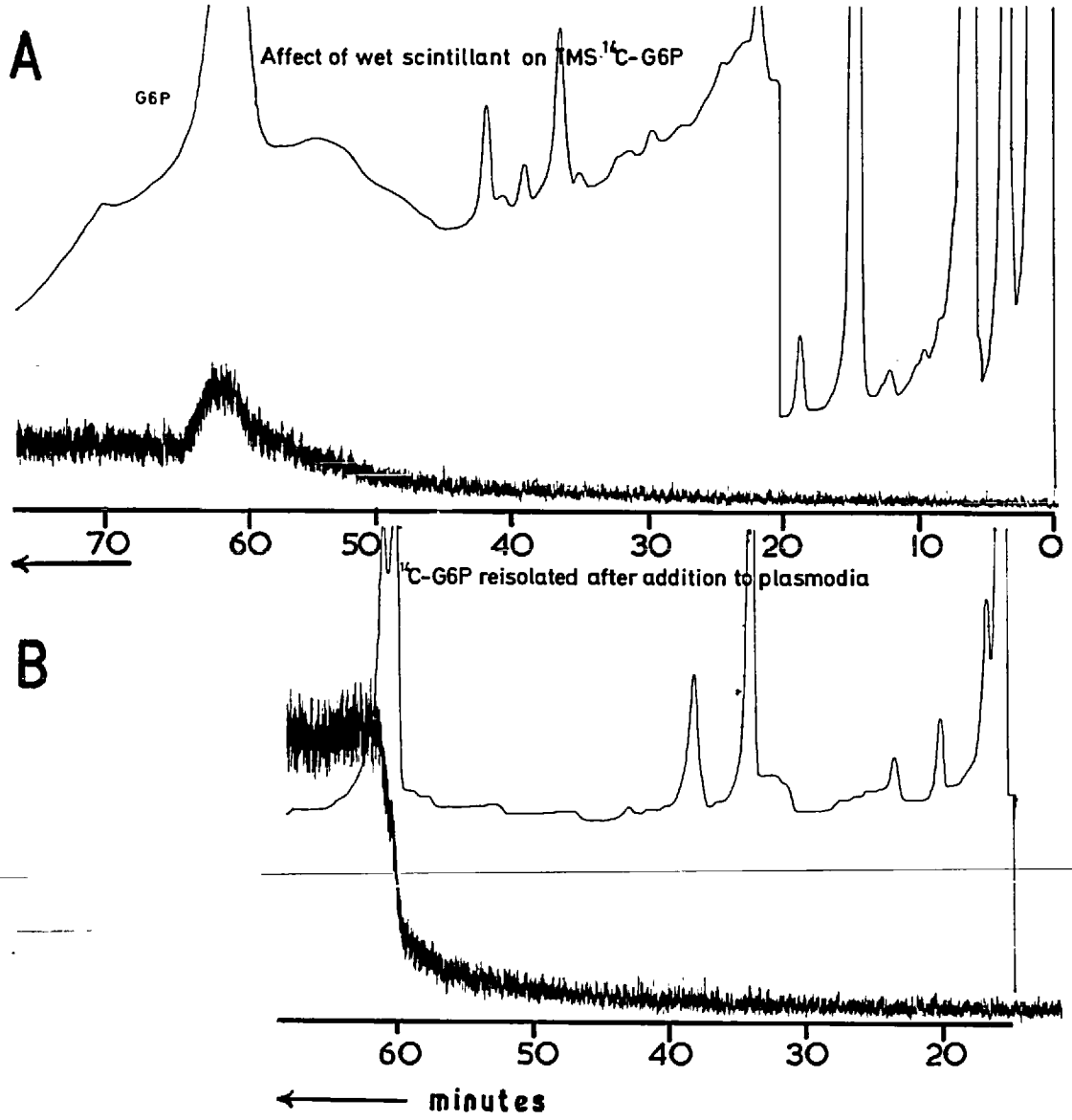

A
G6P
Affect of wet scintillant on TMS-^{14}C-G6P
70
60
50
40
30
20
10
0
^{14}C-G6P reisolated after addition to plasmodia
B
60
50
40
30
20
minutes

Figure 2.5 The effect of 'wet' scintillant on the stability of TMS-G6P

[U-^{14}C]-G6P was added to powdered frozen plasmodia. The plasmodia were extracted with 60% aqueous ethanol, and the extract purified by passage through ion-exchange resin columns (see Chapter 5). After silylation 5 µl were injected onto 3% SE-52 on Chromosorb W (AW-DMCS, 80-100 mesh) column and temperature programmed.

In A freshly made scintillant was added to the cell.

In B the same batch of scintillant was stored over anhydrous sodium sulphate overnight. The disappearance of radioactivity seen in A could be also obtained by adding 2-methoxyethanol.

Figure 2.5. The radioactive derivative TMS-[U-^{14}C]-G6P was hydrolysed by the 'wet' scintillant and came out of solution in the pipework out of sight of the photomultiplier. A normal radiochromatogram is also shown for comparison.

b) The katharometer version of the apparatus

The outlet of the model 44 katharometer was connected to $\frac{1}{8}$" O.D. stainless steel tubing with a Swagelock fitting. A piece of brass, its end tapered to fit the entry to the scintillation cell, was silver soldered to the other end of the tubing. A cylindrical screw with a hexagonal head was fitted onto the tube.

Insulated copper constantan thermocouple wire (Saxonia, gauge 26) joined at one end (silver soldered) was wound around the tubing. Woven glass sleeving was wrapped around this wire, a thermocouple being inserted halfway along. The total resistance of the heating wire was 12 ohms, the temperature being maintained at 200°C. A brass cotton reel was fitted over the cylindrical screw. Copper constantan thermocouple wire was wound around the cotton reel, and after inserting a thermocouple, the whole was covered by woven glass sleeving. The resistance of the heating wire was 16 ohms. Again the temperature was maintained at 200°C. Adjustment of the temperature was crude, taking different tappings off the transformer used, and by varying the length and hence the resistance of the heating wire.

This apparatus is shown in Figure 2.6. The flame ionisation version with stream splitting had the same external appearance.

(contd. on p. 48)

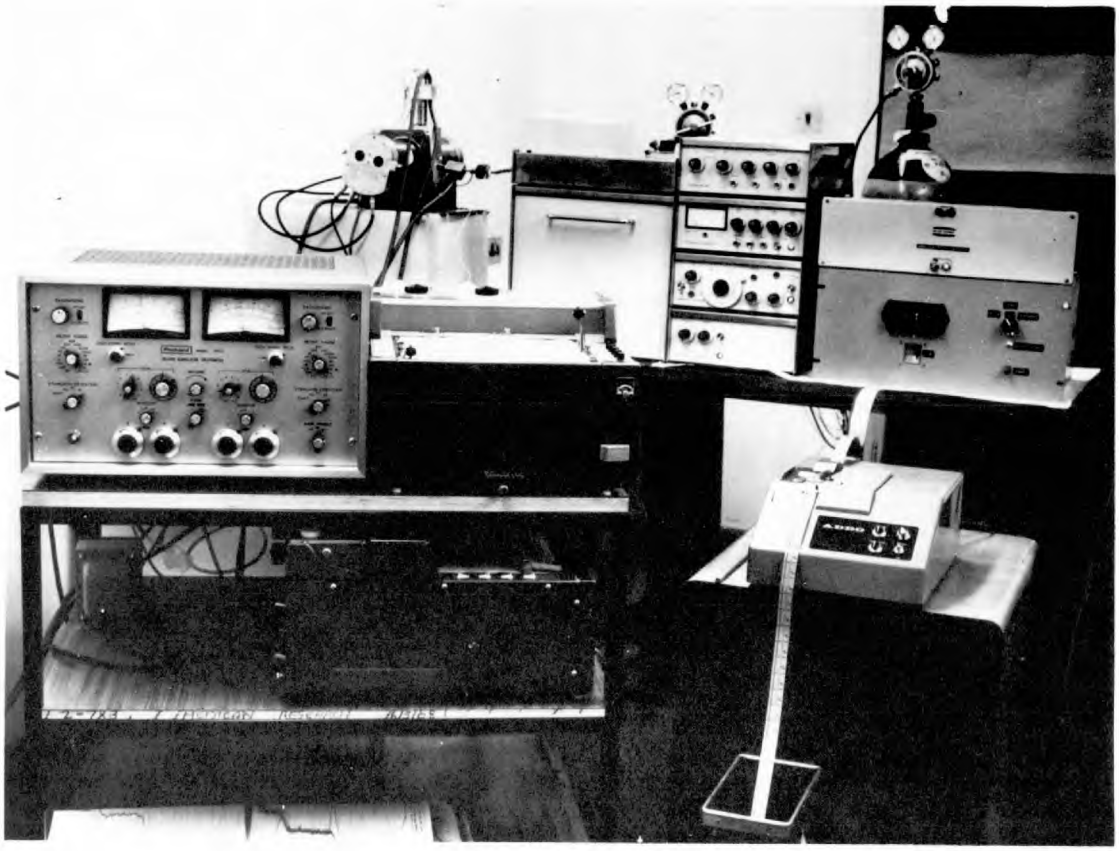


Figure 2.6 The Katharometer version of the radioactive gas liquid chromatography apparatus

This shows the layout of the apparatus used for radioactive gas liquid chromatography. The flame ionisation version looked exactly the same from this viewpoint except that the pipe leading to the scintillation cell came through the wall of the oven rather than from the detector as for the Katharometer version. The pneumatically operated scintillant dispenser was not built when this photograph was taken.

c) The flame ionisation version of the apparatus

A stream splitter was used to prevent all the sample being burnt in the detection system. A split ratio of 1:1 was aimed for. To ensure that the split ratio was unaffected by other pressure effects a large pressure drop was put into both arms of the splitter. Stainless steel tubing (0.0625" O.D., 0.0160" I.D.) was used in the building of the splitter. Stainless steel wire (0.0148" O.D., 8 cms long) was pushed up the two arms of the splitter from the ends to be connected to the splitter. Test measurements showed a pressure drop of 44 cm mercury across this resistance. The ends of the splitter arms were threaded (10 BA).

The stream splitting occurred in a flat hexagonal piece of stainless steel. Holes were drilled perpendicularly into three alternate sides meeting in the centre (see Figure 2.7). This ensured minimum dead volume; the holes were also threaded (10 BA). The threads of the arms were sprayed with teflon to give a gas tight seal when screwed into the sides of the hexagon. The swagelock connector was severed from the glass to metal column connection and the bare stump of 0.0625" stainless steel threaded (10 BA). This was also sprayed with teflon before the hexagon was screwed on. All the connections were checked to see if they were gas tight.

The arm to the flame ionisation detector was 8.5 cm long and connection was made by fitting the swagelock connection removed from the gas to metal connector to the tubing. The other arm was 25 cm long and connection to the scintillation cell was made as described for the katharometer version except that a ring of teflon was compressed between the mating surfaces to ensure a gas and liquid tight seal.

The splitter was heated using the copper-constantan thermocouple wire. Both arms of the splitter were heated since it was found that the flow rate in each varied during temperature programming (see Table 2.1). A thermocouple was included in the arm to the scintillation cell, woven glass sleeving being wound round both arms of the splitter. The resistance of the heating wire was 16 ohms. The cotton reel heater as described earlier was again used. After the mains were stepped down to 25v, a dual channel variable transformer (Regulac-Claude Lyons) was incorporated into the circuit to control the temperature. The temperature of both the heaters was maintained at 250°C.

d) Testing of the F.I.D. version of the radioactive GLC apparatus

The ratio of the flow rates in the two arms of the splitter was shown to vary unless the splitter was heated throughout (Table 2.1). The ratio when both arms were heated to 250°C was 1.19:1 (mass detector: scintillation cell). This was also the ratio of splitting when [1-^{14}C]-n-hexadecane and [U-^{14}C]-Toluene were injected and analysed, either isothermally at 250°C or during a temperature programme. The condensed radioactive materials in toluene in this experiment were measured by withdrawing the contents of the scintillation cell manually with a syringe and then counting in scintillation vials in the Beckman Scintillation Spectrometer (L5-200). This was compared with adding the same amount injected onto the column into the same amount of scintillant present in the scintillation cell in vials and also counting in the Beckman Scintillation spectrometer.

(contd. on p.52)

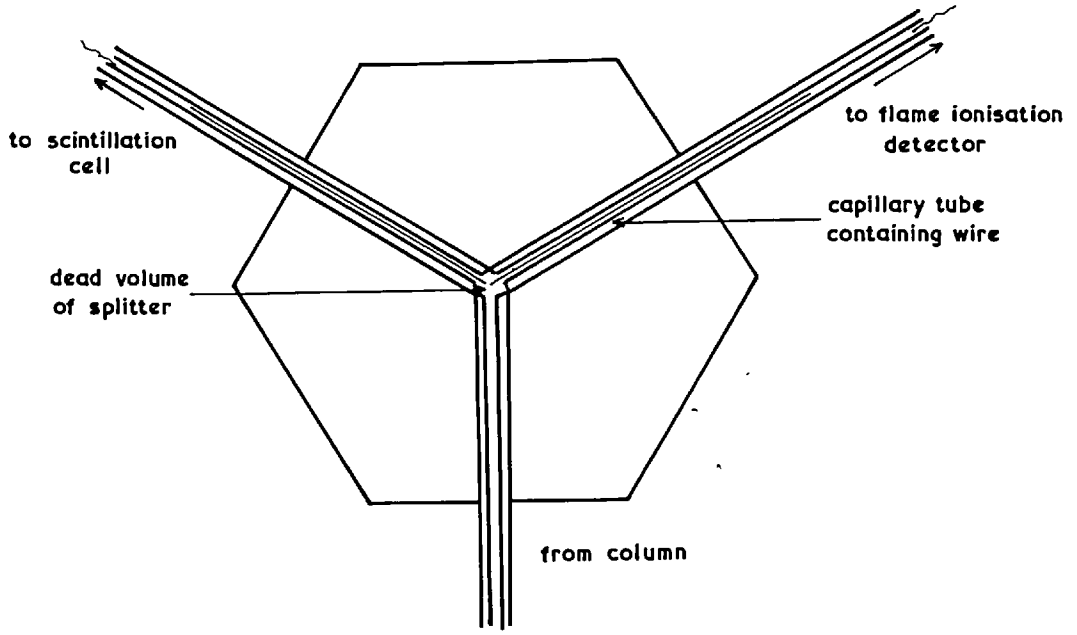

to scintillation cell
to flame ionisation detector
capillary tube containing wire
dead volume of splitter
from column

Figure 2.7 The construction of the stream splitter

A flat hexagonal piece of stainless steel formed the basis of the splitter. Holes were drilled perpendicularly into three facing sides of the hexagon. They were threaded (10 BA). Stainless steel pipe (0.0625" O.D., 0.016" I.D.) which was also threaded (10 BA) formed the arms of the splitter. Stainless steel wire (0.0148" O.D.) was inserted into both arms. The entering stream from the column was also in stainless steel pipe (0.0625" O.D.).

The diagram opposite is not to scale.

Since only very small amounts of sample (5 µl) were injected onto the column the quenching of scintillant did not vary during a chromatographic run. The measured efficiency for the cell varied between 60%-70%. A porportion of the scintillant could not at any one time be seen by the photomultiplier since it was in the pipework, not in the cell. Although loss of toluene during a run was small (about 0.5 ml out of 24 ml) a cooling head was put on the chimney since the measured efficiency was sensitive to the total volume. For volumes greater than 23 ml, the efficiency rises as toluene is lost since a lesser proportion is in the pipework (the volume in the cell remains constant). Below 23 ml the measured efficiency decreases as toluene is lost since a lesser proportion is in the cell. The automatic syringe was consistently able to add 24 ml of scintillant to the cell.

The scintillation data was analysed with a Packard Scintillation Spectrometer (model 3022) which produced a continuous output of counts per minute (i.e. as a ratemeter). The measurements were carried out using a gain of 10% and a window of 50-700. Under these conditions the measured efficiency was 60%. This meant the correction factor to give disintegrations per minute from counts per minute was 3.64. The background was about 100 counts per minute when the data from the two photomultipliers was used with a coincidence circuit. A lower background could have been obtained by encasing the cell in lead and fitting an anticoincidence guard counter. The lowest amount of radioactivity to be measured with an accuracy of less than 10% standard deviation was about 200 cpm, i.e. $\sim$ 800 dpm. In Figure 2.8 TMS derivatives of ^{14}C sucoinate, fumarate, malate and oitrate were separated, the radioactivity associated with each peak being about 1200 dpm. This radiochromatogram shows clearly the need for good chromatographic

(contd. on p.56)

Table 2.1

The variation of the split ratio during temperature programmed analysis

Only the arm to the scintillation cell was heated, the temperature being measured with a thermocouple with the reference junction in melting ice (0°C). The temperature of the other arm was given as the temperature of the oven measured with a thermometer. The temperature was allowed to stabilise in the oven for 15 minutes before measurement of flow rate was carried out. These measurements were made in triplicate with a soap bubble flow meter.

To the F.I.D.		To the scintillation cell		Total	Split
Temperature in °C	Flow rate mls/min	Temperature in °C	Flow rate mls/min	Flow rate mls/min	Ratio
25	25.5	25	27.3	52.8	1.07
25	17.1	220	34.3	51.4	2.01
70	20.3	220	30.8	51.1	1.52
120	21.8	220	29.6	51.4	1.36
170	23.5	220	27.8	51.4	1.19
200	23.5	220	27.9	51.4	1.19

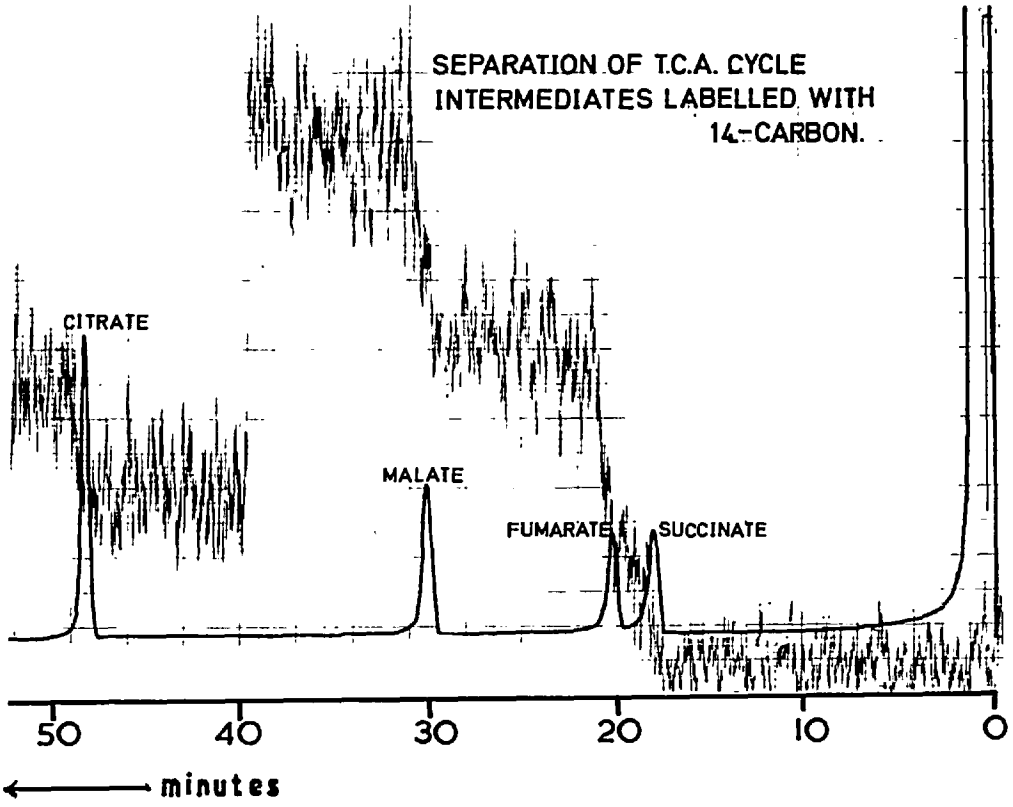

SEPARATION OF T.C.A. CYCLE
INTERMEDIATES LABELLED WITH
14-CARBON.
CITRATE
MALATE
FUMARATE
SUCCINATE
50
40
30
20
10
0
minutes

Figure 2.8 Separation of trimethylsilyl ester-trimethylsilyl ether derivatives of some ^{14}C-intermediates using the radioactive GLC apparatus (F.I.D. version)

The derivatives of citrate, fumarate, succinate and malate were prepared using pyridine:HMDS:TMCS (10:2:1, by vol). A 3% SE-52 on Chromosorb W (AW-DMCS, 80-100 mesh) column was used for the chromatographic separation. The temperature programme 70-180°C at 2°C per minute was used.

separation of the compounds under study. TMS-succinate and TMS-fumarate are separated according to their mass trace, but the radio-activity record is difficult to interpret to give good accuracy. The longer the peak can remain in the cell without further peaks being eluted the better.

9) Thin layer chromatography of sugar phosphates and their derivatives

The materials used were Kieselguhr G (nach Stahl) and Whatman CC41 cellulose powder. To make five 20 x 20 cm plates (250 µm thick layer) 25 g of solid was taken and was shaken with 56 ml distilled water (sufficient to give the correct slurry consistency). For silver nitrate impregnated plates 22.5 g of Kieselguhr G (nach Stahl) was taken and shaken with 50 ml distilled water containing 2.5 g silver nitrate. The slurry was spread onto clean glass plates with a Shandon Unoplan spreader. The plates were removed from the spreader immediately after spreading, and were tapped to remove any lines made by marks on the spreader. The plates were left on a flat surface to dry. When a matt finish could be seen they were transferred to an oven at 120°C and left to activate for 90 minutes. 20 x 5 cms plates were made similarly.

After spotting the plates and drying each spot with a cold draught, they were placed in a Shandon Chromatank containing the appropriate solvent. Filter paper lined the walls of the tank, and the solvent was added by pouring onto the filter paper at least 20 minutes before the plates were ready.

Solvent systems used:

Solvent A n-propanol:0.880 ammonium hydroxide:water 6:3:1 by vol

Solvent B ethyl acetate:iso-propanol:water 65:24:11 by vol

Solvent C benzene:ethanol (proportions specified each time)

Solvent D petroleum ether (40-60°C):diethyl ether 9:1 v/v

Spray reagents for locating materials on TLC plates:

1. 2.5M H_2SO_4 - for all organic materials - the plate was heated at 150°C for 10-20 minutes.
2. Molybdate reagent (Hanes & Isherwood, 1949). Plates were developed under an ultra-violet lamp.
3. Aniline-Diphenylamine (Buchan & Savage, 1952). Kieselguhr G plates were heated at 130-140°C for 15 minutes, longer if necessary. Cellulose plates were not heated above 100°C since the background became strong.
4. Aniline Phthalate (Fernez & Stoffyn, 1959)
   TLC plates were heated at 105°C for 5 minutes.
5. Silver nitrate (Trevelyen et al, 1950).

Saturated silver nitrate solution (1 ml) was added to acetone (200 ml). Distilled water was added to just dissolve all the white precipitate. After spraying, the plates were heated at 45-50°C. They were then sprayed with 0.5% (w/v) potassium hydroxide in methanol. The spots were fixed with 2.5% (w/v) sodium thiosulphate solution.

The radioactive scanning of TLC plates

20 x 5 cm plates were used so as to fit the track on the scanner (Nuclear Chicago, Actigraph II model 1025). A scanning rate

of 30 cms/hour was used for analysis. A rate of 100 cms/hour was used to determine on which sensitivity range the analysis was to be carried out.

10) Colorimetric Analysis

a) The determination of phosphate

1. Inorganic phosphate (Martin & Doty, 1949) a minor modification of that of Berenblum & Chain (1938)

The following reagents were used:

A 5M H_2SO_4

B 10% ammonium molybdate

C iso-butanol:benzene 1:1 v/v

D 10% stamous chloride in concentrated HCl, diluted before use with 0.5M H_2SO_4

E 3.2% (v/v) H_2SO_4 in ethanol.

To 3 ml of sample (if necessary diluted with water) A (0.5 ml) was added followed by C (5.0 ml) and B (0.5 ml). The tube was vigorously shaken and then centrifuged (1000 g, 2 mins). An aliquot (1 ml) of the upper layer was taken and was diluted with E (4.0 ml). D (0.5 ml) was added and the tube shaken vigorously. The absorbance of the blue solution was read at 625 nm against a blank prepared similarly containing water (3 ml) instead of the sample. A standard curve was prepared using potassium dihydrogen phosphate. Each analysis was carried out in duplicate.

2. Total phosphate (Fiske & Subbarow, 1925)

The material for analysis was hydrolysed with 5M H_2SO_4 (0.5 ml) in a digestion flask at 160°C for 3 hours. Hydrogen peroxide (2 drops) was added and heating continued for a further 90 minutes.

To each digest water (4.4 ml) was added followed by 5% ammonium molybdate (0.2 ml) and the Fiske-Subbarow reagent (0.2 ml). This reagent was prepared by adding sodium sulphite (0.1 g) and 1-amino-2-naphthol-4-sulphonic acid (0.1 g) to 15% sodium metabisulphite solution (40 ml), stirring for 30 minutes and finally filtering to remove fine particles.

The incubation mixture was heated in a water bath (at 100°C) for 7 minutes. The absorbance of the blue green colour was read at 830 nm against a blank prepared similarly containing water instead of the sample. A standard curve was prepared using potassium dihydrogen phosphate.

b) The enzymatic determination of glucose using glucose oxidase

Medium samples were not deproteinised before assay. Glycogen hydrolysates were neutralised to pH 6 with potassium hydroxide. Each sample was diluted where necessary to give a concentration between 20-100 µg glucose/ml.

The method of assay using an autoanalyser (Technicon) procedure was that of Marks & Lloyd (1963). The protein precipitation step was omitted. The sample was mixed with an enzyme-dye reagent, and the colour produced measured at 660 nm. The enzyme-dye reagent was freshly prepared before use. Its composition was as follows:

0.05M sodium acetate buffer pH 5.0	800 ml
peroxidase (20 mg in 500 ml of the above buffer)	50 ml
o-tolidene (1% solution in absolute ethanol)	10 ml
fermcozyme (glucose oxidase mixture)	10 ml

This mixture was made up to 1 litre with the buffer.

A series of standard glucose solutions (5, 10, 20, 40, 60, 80, 100 µg per ml) were prepared for each analysis and run before and after analysis of the unknowns.

CHAPTER 3.

THE PREPARATION OF VOLATILE DERIVATIVES OF THE COMPOUNDS TO BE STUDIED BY GAS CHROMATOGRAPHY

At the outset the volatile derivatives of the carbohydrate intermediates whose preparation had been previously described in the literature were investigated.

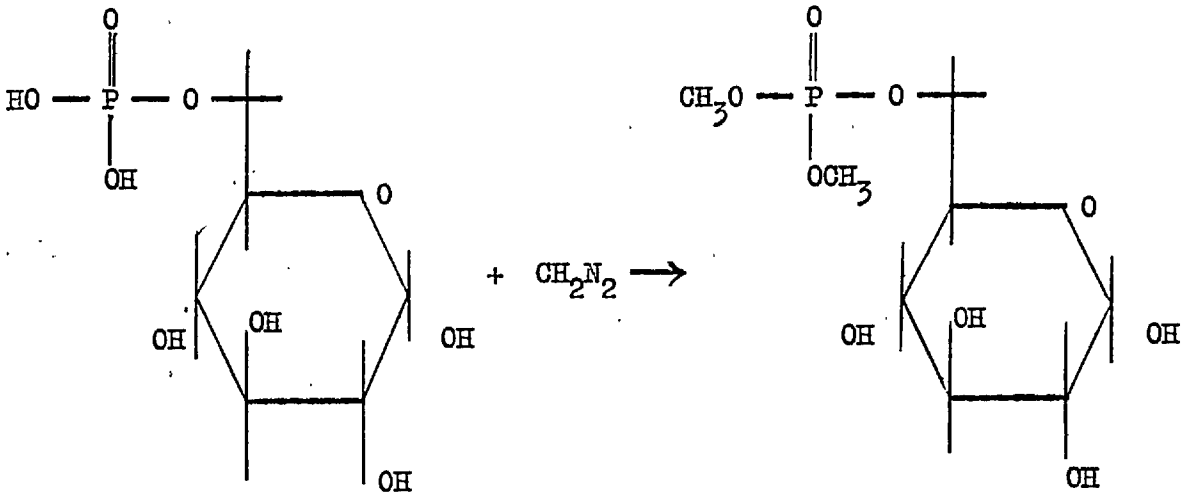
A. The dimethyl ester-trimethylsilyl ether derivative of sugar phosphates

This was the first volatile derivative reported for the sugar phosphates (Wells et al, 1964). There has however been no further application of the method. The investigations reported below indicated that this derivative was only suitable for the quantitative analysis of G6P, and even then it gave rise to minor peaks which would have invalidated radioactive measurements. For F6P quantitative formation of the derivative was not possible. Unsuccessful attempts were made to find a method of preparation for the derivative of G1P.

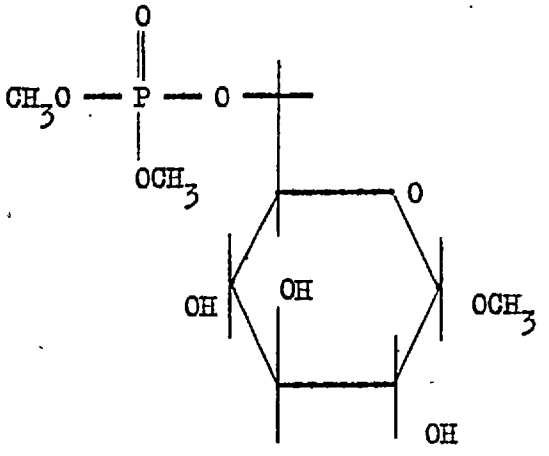
1) The products of the methylation of fructose-6-phosphate and glucose-6-phosphate with diazomethane

The methylation with ethereal diazomethane of the free acids of G6P and F6P (prepared either by passing an aqueous solution of the disodium salts down a short column of AG 50(H^+) resin, or by shaking with the same resin in methanol) led to multiple products as judged by TLC of the reaction mixture (see Figure 3.1). For G6P two spots were observed when the plate was sprayed with 2.5M sulphuric acid, whereas only the less mobile spot was visible when an identical plate was sprayed

with silver nitrate spray reagent (see Methods 2.9). This effect was also observed when the methylation products of G1P were analysed (see Section 3.4). The more mobile spot could be a non-reducing sugar, i.e. blocked in the C-1 position by a methyl group. This would be α- or β-methyl glucoside-6-dimethyl phosphate.



and



When [U-^{14}C]-G6P was used to investigate this reaction radioactive TLC scanning of the spots indicated that both were radioactive (see Figure 3.2).

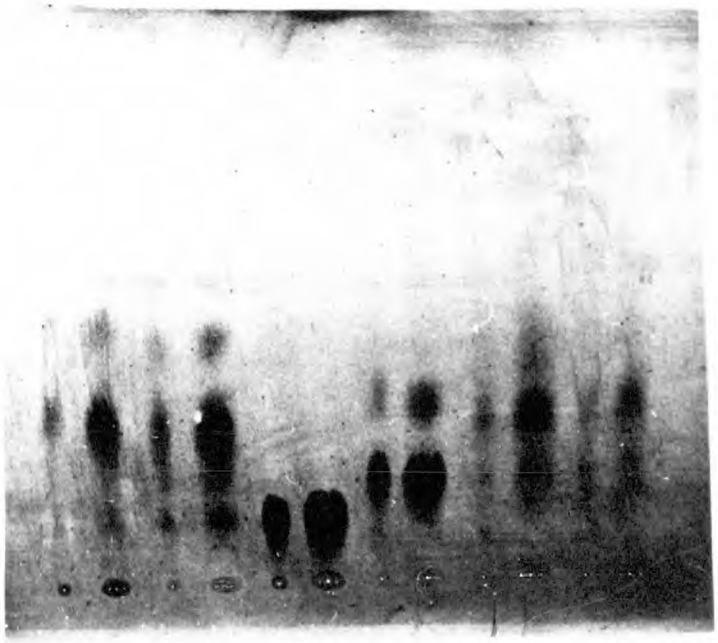
(contd. on p.65)

A



A A A A B B C C D D

B



A A A A B B C C D D D D

Figure 3.1 Thin layer chromatography of the products of methylation of sugar phosphate with diazomethane

Each sugar phosphate was converted to its free acid with AG 50 (H^+ form) resin in methanol. The resulting solutions were treated with ethereal diazomethane. The reaction mixtures were spotted onto 20 x 20 cm Kieselguhr G plates (250 μm thick layer) at two concentrations, one three times the other. The plates were developed twice with benzene:ethanol (4:1 v/v), drying carefully in between each run. The plates were sprayed with silver nitrate spray reagent (A) and 2.5M sulphuric acid (B) respectively and treated as described in Methods.

A Glucose-1-phosphate

B Glucose reference

C Glucose-6-phosphate

D Fructose-6-phosphate

When the methylated G6P reaction mixture was taken to dryness and then treated with pyridine:hexamethyldisilazane (HMDS):trimethylchlorosilane (TMCS) (10:2:1 by vol) at room temperature, GLC analysis using 3% SE-30 on Chromosorb W showed two major peaks with a shoulder preceding them (see Figure 3.3). When the two spots on the TLC mentioned above were eluted separately with methanol and then silylated, it was found that the less mobile spot (the reducing spot) gave rise to the two major peaks and the more mobile spot (non-reducing) to the shoulder.

For F6P the methylation reaction mixture gave rise to many spots on the TLC plate. This after silylation gave rise to one peak with a preceding shoulder when analysed by GLC (see Figure 3.4). However it was likely that the observed peak was a mixture of unseparated peaks.

(contd. on p.72)

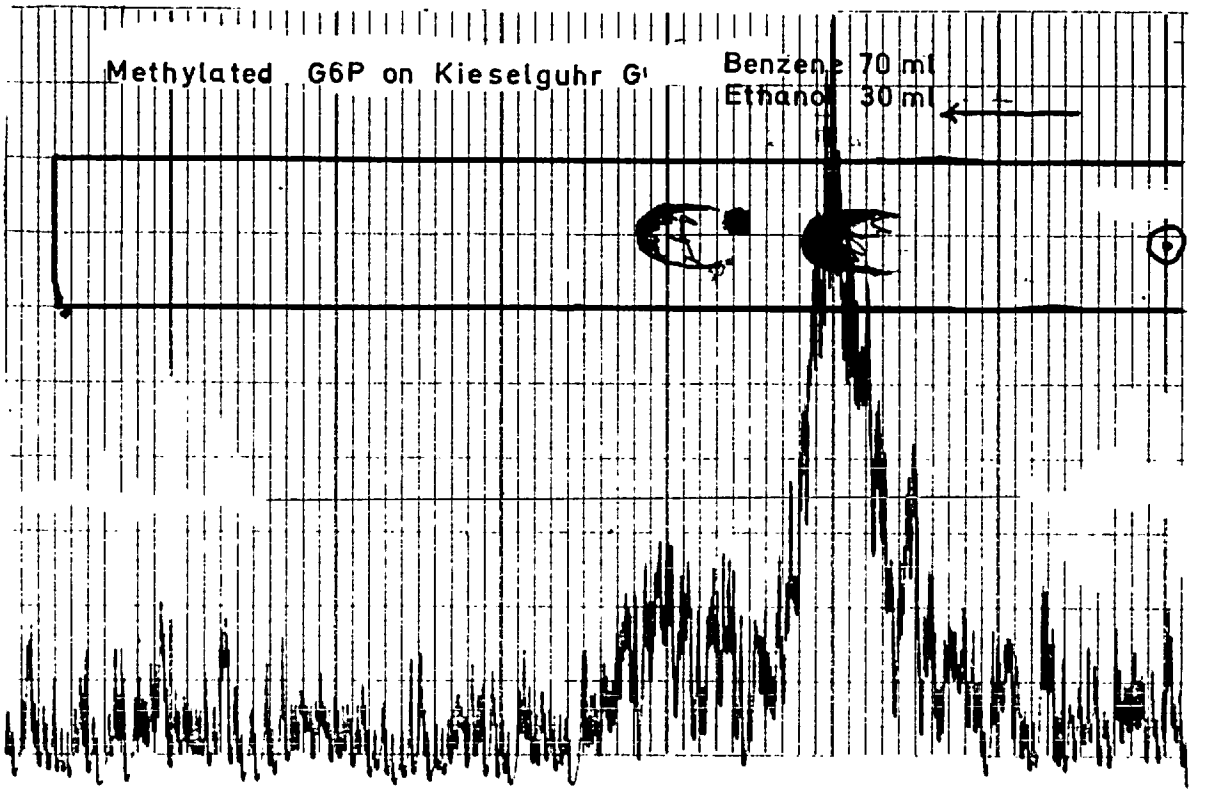

Methylated G6P on Kieselguhr G
Benzene 70 ml
Ethanol 30 ml

Figure 3.2 Radioactive scanning of the TLC plate on which the separation of the products of the methylation of G6P with diazomethane had been carried out

[U-^{14}C]-G6P was converted to the free acid with AG 50(H^+) resin in methanol. The resulting solution was treated with ethereal diazomethane. The reaction mixture was spotted onto a 20 x 5 cm glass plate coated (250 μm thick layer) with Kieselguhr G. The plate was developed with benzene:ethanol (7:3 v/v). It was scanned for radioactivity at 12"/hour. After scanning it was sprayed with aniline diphenylamine reagent (Methods) to locate the spots. The plate after location was compared with the radioactivity scan as shown opposite.

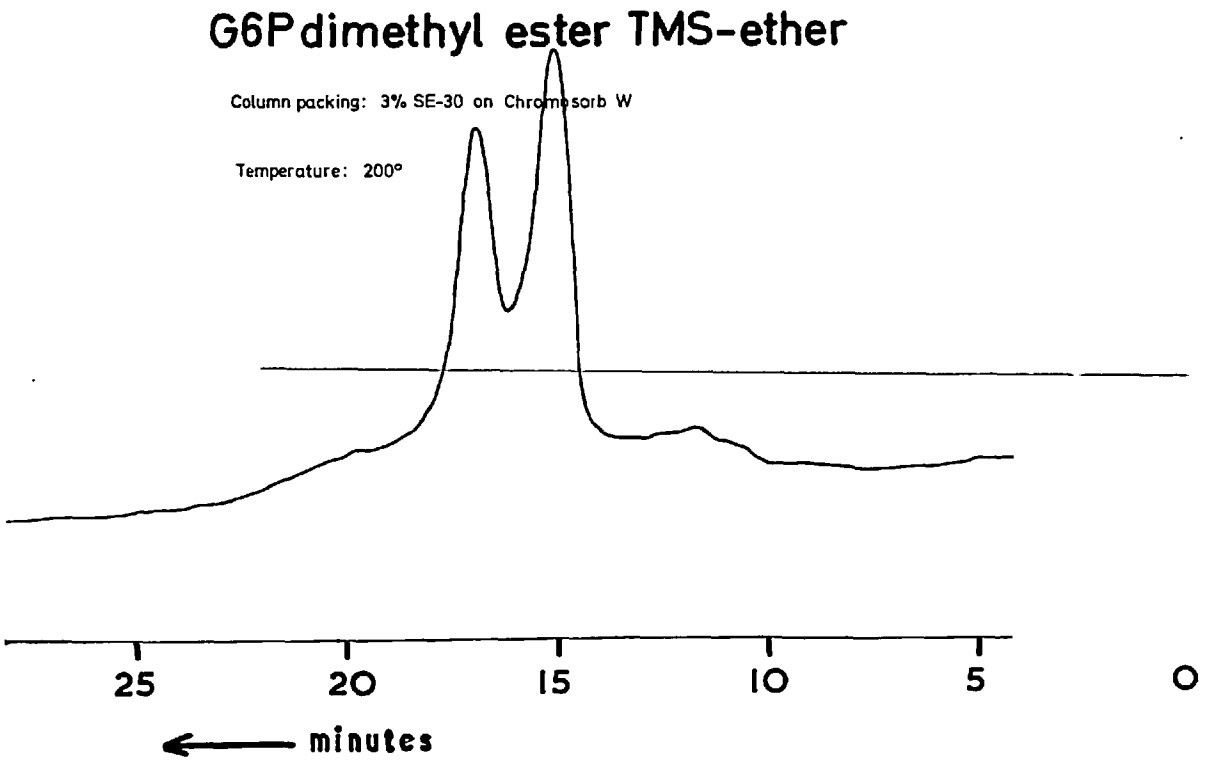

G6P dimethyl ester TMS-ether
Column packing: 3% SE-30 on Chromosorb W
Temperature: 200°
25
20
15
10
5
0
minutes

Figure 3.3 Gas chromatography of the products of the treatment of G6P with diazomethane, and their subsequent silylation

Disodium G6P was shaken in methanol with AG 50(H^+ form) resin to form the free acid. This solution was treated with ethereal diazomethane until there was a permanent yellow colour. The solution was evaporated to dryness under reduced pressure and then treated with pyridine:HMDS:TMCS (10:2:1 by vol), 0.1 ml 5 µl aliquots were analysed using a 3% SE-30 on Chromosorb W (AW-DMCS, 80-100 mesh) column at 200°C.

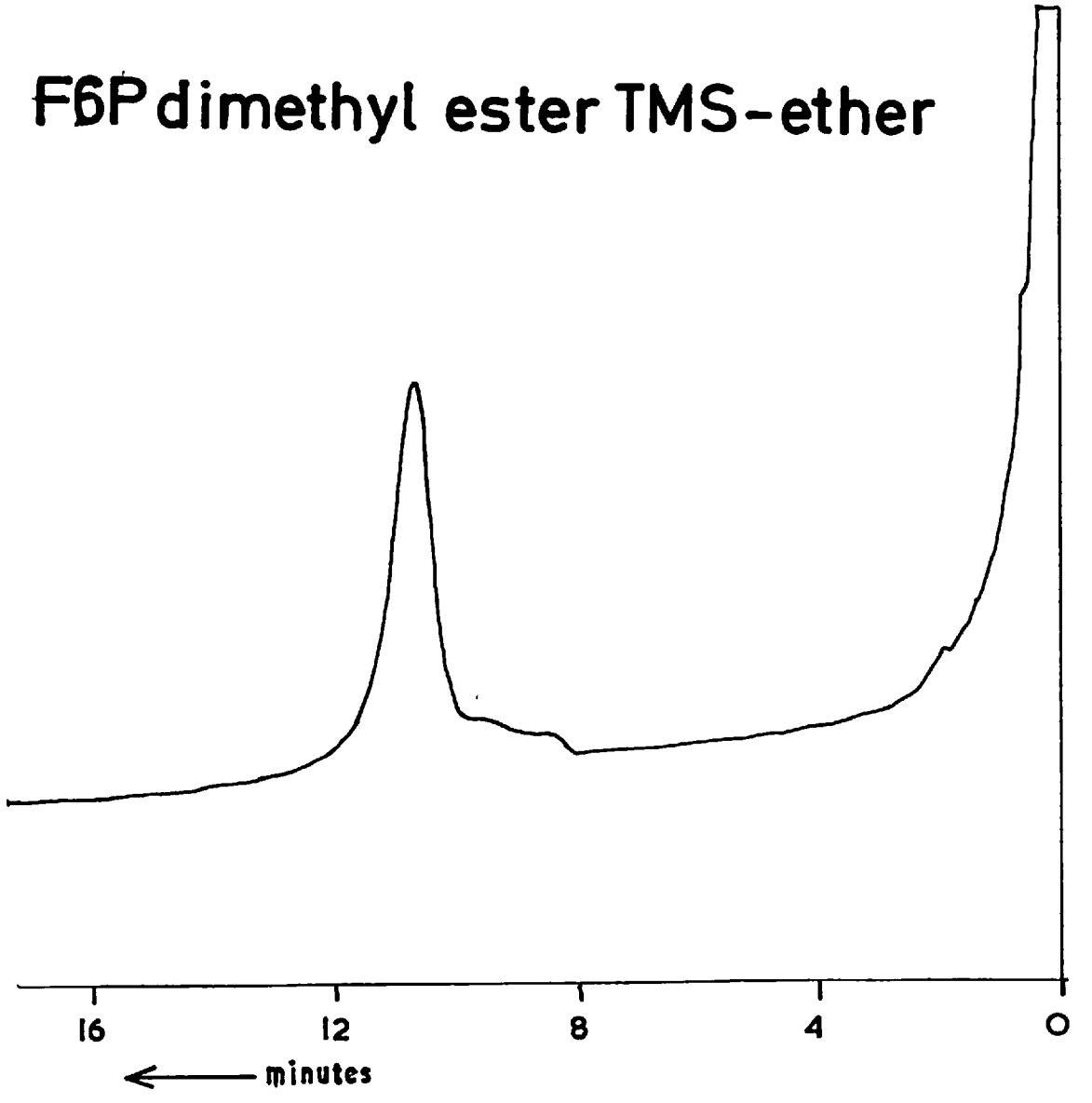

F6P dimethyl ester TMS-ether
16
12
8
4
0
minutes

Figure 3.4 Gas chromatography of the products of the treatment of F6P with diazomethane and their subsequent silylation

Disodium F6P was treated in a similar fashion to G6P (Figure 3.3). An aliquot was injected onto a 3% SE-30 on Chromosorb W (AW-DMCS, 80-100 mesh) column, 5' x $\frac{1}{4}$", at 200°C.

2) The Quantitative Analysis of G6P and F6P by GLC

Since the formation of the derivative for GLC was a two stage process, the reproducibility of both stages was investigated. Four separate solid samples of G6P and F6P were taken and methylated and silylated individually. The response per µg of G6P and F6P injected onto the GLC column was determined for each sample (see Table 3.1). G6P gave a reproducible response whereas F6P varied from sample to sample, and in each sample with respect to time.

In the next experiment the four samples were combined and methylated together. The methylation mixture was divided into four equal parts, and each silylated. The response per µg was determined (see Table 3.2). Again G6P gave a reproducible response. F6P, whilst still varying with time, was reproducible between different samples at each time point. A similar experiment was carried out starting with aqueous solutions of G6P and F6P, methylating the combined solutions and dividing again prior to silylation. The results are given in Table 3.3. Once again G6P gave a reproducible response, whereas that for F6P was more variable.

Thus G6P could be analysed as its dimethylester-trimethylsilyl-ether derivative by GLC starting with a pure aqueous solution and quantitative data be obtained. F6P was not converted to its derivative reproducibly.

(contd. on p.76)

Table 3.1

The reproducibility of formation of derivatives of G6P and F6P - (separate samples)

Each sugar phosphate sample was converted to its free acid in methanol and treated with ethereal diazomethane. It was taken to dryness and reacted with pyridine:HMDS:TMCS (10:2:1 by vol).

Chromatography was carried out on 3% SE-30 on Chromosorb W (AW-DMCS, 80-100 mesh) at 200°C, injecting 5 µl aliquots. Samples were silylated 45 minutes before the first chromatographic run. For F6P the final analysis was carried out the following day. Areas of peaks were determined by cutting out and weighing them.

Sample	Peak area/µg G6P injected	Sample	Peak area/µg F6P injected
1	1.55, 1.68	1	1.43, 1.72, 1.92
2	1.53, 1.47	2	0.31, 0.36, 0.46
3	1.62, 1.53	3	1.67, 1.77, 1.78
4	1.59, 1.57	4	0.90, 0.95, 1.16

Table 3.2

The reproducibility of the silylation step in the formation of derivatives for G6P and F6P

G6P and F6P were methylated with ethereal diazomethane and then divided into 4 samples, each of which was separately silylated. Chromatography was carried out on 3% SE-30 on Chromosorb W (AW-DMCS, 80-100 mesh) at 200°C, injecting 5 µl aliquots. Samples were silylated 45 minutes before the first chromatographic run. For F6P the final analysis was carried out the following day. Areas of peaks were determined by cutting out and weighing them.

Sample	Peak area/µg G6P injected	Sample	Peak area/µg F6P injected
1	1.84, 1.90, 1.80	1	1.16, 1.41, 1.55
2	1.88, 1.88	2	1.27, 1.61, 1.61
3	1.88, 1.83	3	1.30, 1.41, 1.57
4	1.85, 1.87	4	1.39, 1.39, 1.66

Table 3.3

The reproducibility of derivative formation of aqueous solutions of G6P and F6P

4 equal samples were taken of aqueous samples of F6P and G6P. Each was taken to dryness under vacuum. The free acids were dissolved in methanol and reacted with ethereal diazomethane. The reaction mixtures were taken to dryness and each treated with pyridine: HMDS:TMCS (10:2:1 by vol).

Chromatography was carried out on 3% SE-30 on Chromosorb W (AW-DMCS, 80-100 mesh) at 200°C, injecting 5 µl aliquots. Samples were silylated 48 hours before chromatography. For F6P the final analysis was carried out the following day. Areas of peaks were determined by cutting out and weighing them.

Sample	Peak area/µg G6P injected	Sample	Peak area/µg F6P injected
1	1.20, 1.29, 1.23	1	0.78, 0.70, 0.82
2	1.19, 1.20	2	0.48, 0.54, 0.66
3	1.17, 1.20	3	0.65, 0.65, 0.75
4	1.16, 1.16, 1.12, 1.19	4	0.97, 0.95, 1.06

3) Investigations on the products of the reaction between glucose-1-phosphoric acid and ethereal diazomethane, and their subsequent silylation

When G1P was treated as described by Wells et al (1964) for the preparation of the dimethyl ester-trimethylsilyl ether derivative of other sugar phosphates, GLC analysis of the product indicated TMS-α-D-glucose as the major peak. This was to be expected since concentration of an aqueous solution of the free acid of G1P would cause it to catalyse its own hydrolysis to glucose and phosphoric acid.

It was evident from the work of Wells et al (1964) that the free acids of sugar phosphates were soluble in alcohols. It was hoped that the rate of hydrolysis of G1P would be reduced in these solvents. The most effective way of preparing the free acid was to shake the disodium salt in the alcohol used with AG 50 resin in the hydrogen form. Tests were carried out with disodium salt of [U-^{14}C]-G1P which showed that all the G1P was dissolved by this treatment in methanol (NB the disodium salt was precipitated from an aqueous solution by methanol at a percentage composition of 55%).

The stability of the free acid so prepared in methanol at 0°C was investigated. Aliquots were removed from a methanol solution after periods up to 60 minutes. The aliquots were neutralised with ammonium hydroxide, and TLC analysis on cellulose (solvent A - see Methods) carried out. No free glucose could be detected, or free inorganic phosphate could be seen when the plates were sprayed with aniline-diphenylamine reagent (for sugars), or the molybdate reagent (for phosphate), there being a single spot of glucose-1-phosphate.

Glucose oxidase assay of the 60 minute sample showed a glucose content of 1% of the theoretical amount that could be released on complete hydrolysis. Spectrophotometric assay for inorganic phosphate indicated a small amount of hydrolysis (5% after 60 minutes, see Figure 3.5). Since dissolving G1P in methanol and transferance of the solution to another tube took only two minutes when carrying out a methylation, the extent of hydrolysis before addition of ethereal diazomethane was very small.

Addition of ethereal diazomethane to the methanolic solution of G1P produced a vigorous reaction, and TLC analysis on cellulose (solvent A, see Methods) showed that no G1P salt remained. However, when the reaction mixture was subsequently silylated, GLC analysis again showed TMS-α-D-glucose to be present as well as other compounds of shorter retention times (see Figure 3.6).

4) The estimation of the glucose content of the products of reaction between G1P and diazomethane

The amount of glucose present in the methylation reaction mixture was assayed spectrophotometrically using glucose oxidase, and also by GLC analysis after silylation. The results of these assays for eight separate samples are shown in Table 3.4. The large difference between these assays, 60% for glucose oxidase and 30% for GLC, was consistent. The GLC data was confirmed in a separate experiment (Table 3.5). Sample 1 in this set of data gave rise to a GLC chromatogram quite unlike the other samples. A peak of much greater retention time was observed (see Figure 3.7). The percentage glucose content of this sample was low (11%) compared with all other samples. This result could be repeated by adding excess ammonia after the addition

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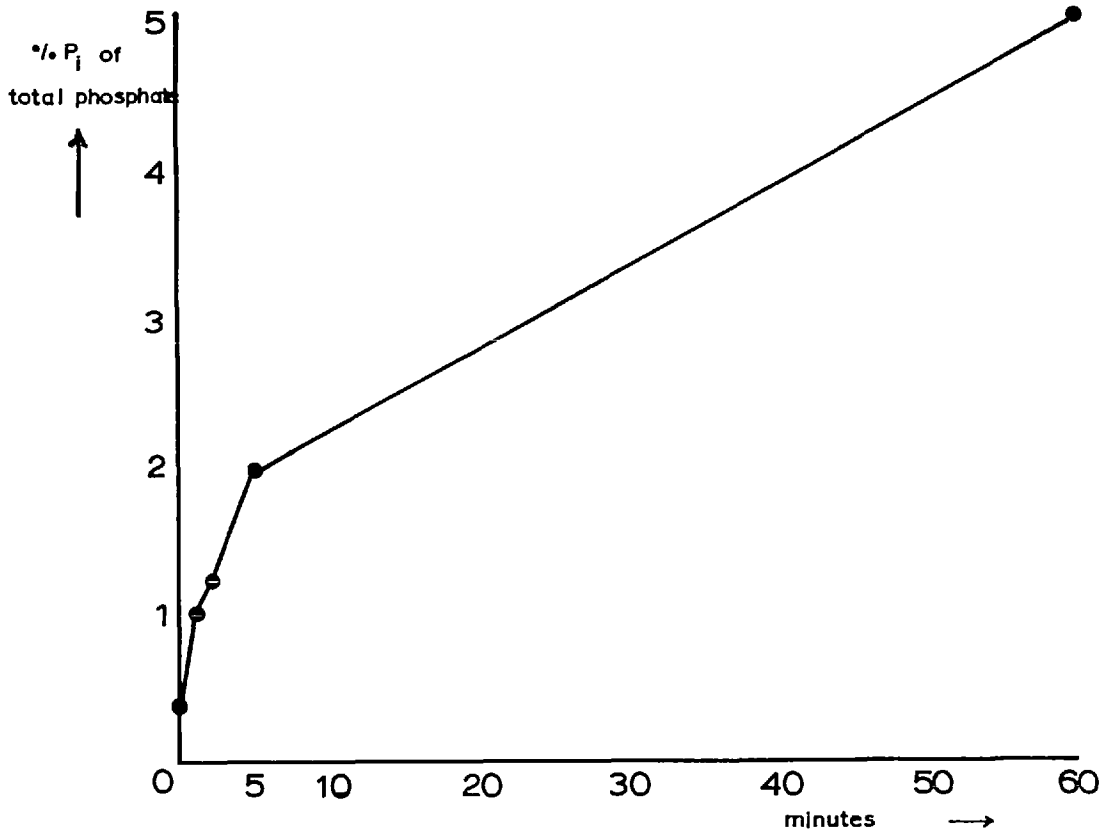

% P_i of
total phosphate
5
4
3
2
1
0
5
10
20
30
40
50
60
minutes

Figure 3.5 Hydrolysis of glucose-1-phosphoric acid to inorganic phosphate

A solution of glucose-1-phosphoric acid in methanol was prepared by shaking the disodium salt with AG 50 (H^+ form) resin in methanol. The concentration was 10 mg G1P per ml. 1 ml aliquots were removed at the times indicated and were immediately neutralised with 0.880 ammonium hydroxide. This solution was tested for inorganic phosphate by the method of Martin & Doty (1949). A further 1 ml portion was analysed for total phosphate (Fiske & Subba Row, 1925). This value obtained was made 100% and the values for the other aliquots related to it.

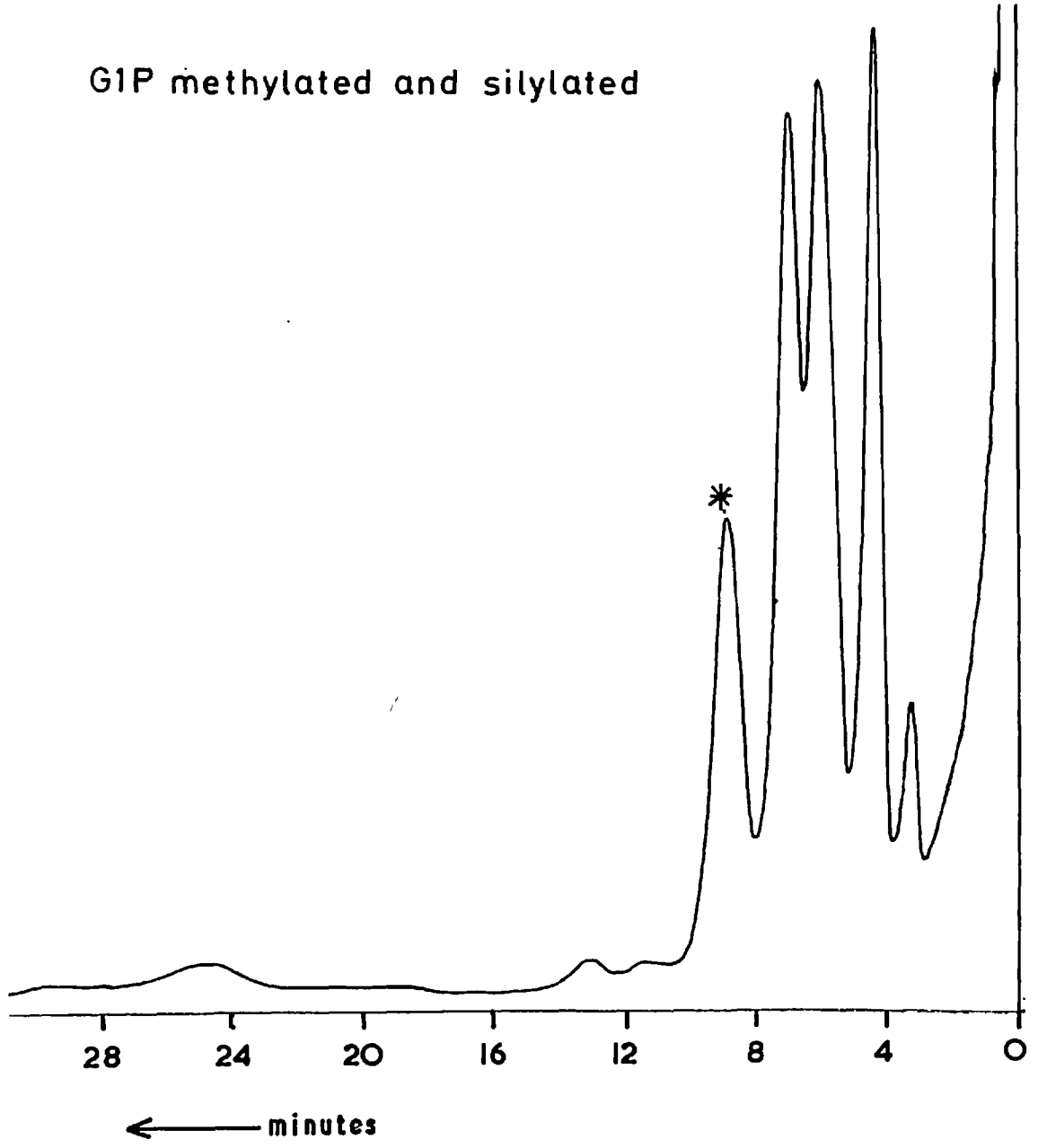

G1P methylated and silylated
*
28
24
20
16
12
8
4
0
minutes

Figure 3.6 Gas chromatography of the products of the treatment of G1P with diazomethane and silylation reagents

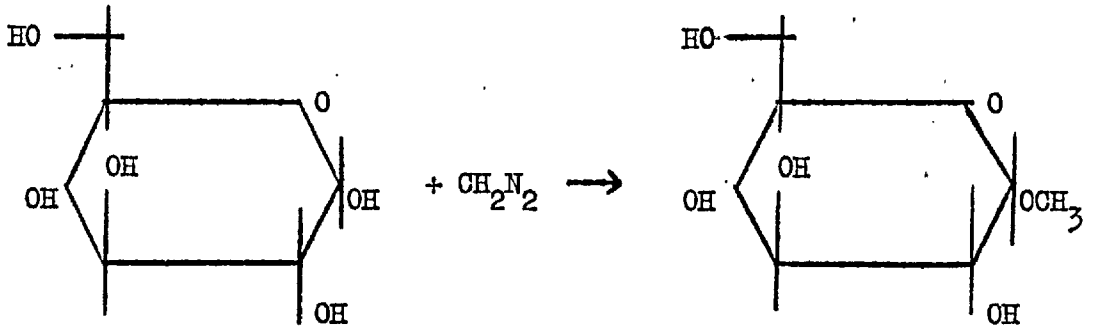
The disodium salt of ∝-G1P was shaken in methanol with AG 50 (H^+) resin to form a methanolic solution of the free acid. Ethereal diazomethane was added until a permanent yellow colour remained. The solution was taken to dryness and reacted with pyridine:HMDS:TMCS (10:2:1 by vol). 5 µl aliquots were injected onto a 3% SE-30 on Chromosorb W (AW-DMCS, 80-100 mesh) column at 180°C.

* This peak corresponds to TMS-∝-D-glucose

of ethereal diazomethane. However the large number of peaks in the chromatogram prohibited the use of this method for quantitative analysis.

TLC results indicated that both the estimations of the percentage glucose content of the G1P methylation reaction mixtures were high. Chromatography was carried out on Kieselguhr G (nach Stahl) (solvent: benzene:ethanol 4:1 v/v). When silver nitrate spray reagent was used a spot of R_F the same as glucose was the major spot. However when an identical plate was sprayed with 2.5M sulphuric acid and then charred, the spot corresponding to glucose was minor compared to a spot of greater R_F (see Figure 3.1). This spot had the same R_F as ∝-methylglucoside. Using [U-^{14}C]-labelled G1P, radioactive-TLC scanning of a similar plate (20 cms x 5 cms) showed that the major radioactivity was associated with the major spot (R_F similar to ∝-methylglucoside) and little or no radioactivity with glucose (see Figure 3.8).

Kuhn and Baer (1953) reported that glucose could react with diazomethane in a methanol:water mixture (9:1) to give predominantly ∝-methylglucoside.



Thus if glucose were released during methylation, formation of ∝-methyl-glucoside would be possible. Further methylation of the sugar ring could also occur. This would account for the spots of greater R_F than ∝-methylglucoside which were observed.

(contd. on p.88)

Table 3.4

Glucose content in reaction mixture after methylation of G1P by diazomethane (8 separate samples)

10 mg G1P disodium salt was reacted in methanol with AG 50 resin (H^+ form). The resulting solution was reacted with excess ethereal diazomethane. A 1 ml aliquot was diluted to 10 ml with water, and analysed for glucose content by the glucose oxidase procedure. Another 1 ml aliquot was taken to dryness and silylated with 1 ml pyridine:HMDS:TMCS (10:2:1 by vol). The glucose content was determined by gas chromatographic analysis on 5% SE-30 on Chromosorb W (AW-DMCS, 80-100 mesh) at 180°C. The results of both analyses were related to the glucose that theoretically was present as G1P initially.

Sample No.	% glucose formed	
	Glucose oxidase	GLC
1	41.1	21.9
2	62.7	33.4
3	59.6	31.5
4	65.8	34.0
5	60.4	33.2
6	48.2	30.4
7	57.8	32.9
8	51.1	33.1

Table 3.5

Glucose content after methylation of G1P by diazomethane (4 separate samples)

Sample No.	% glucose formed, by GLC
1	11.1
2	28.8
3	31.4
4	32.4

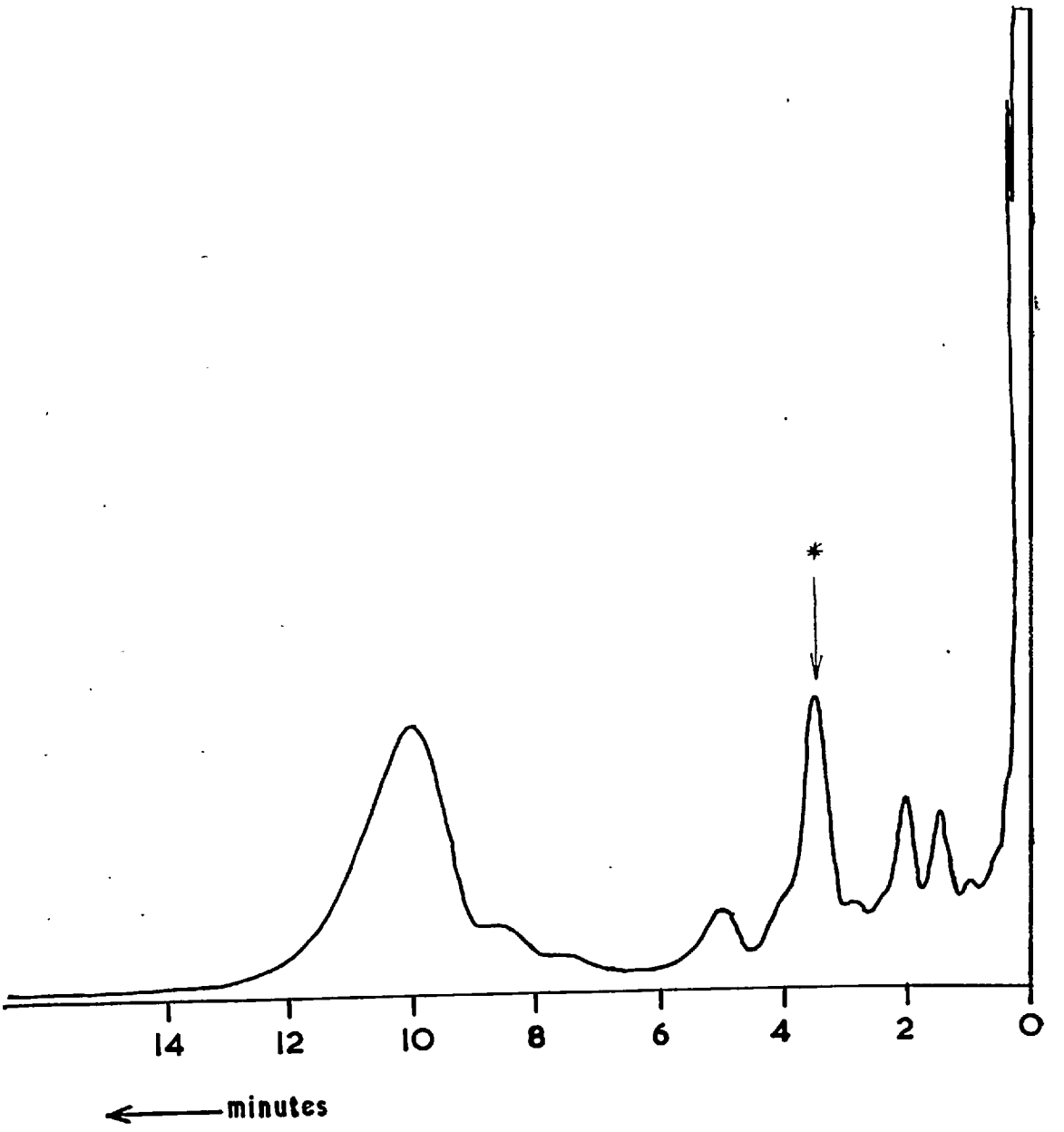

*
14
12
10
8
6
4
2
0
minutes

Figure 3.7 An unusual chromatogram obtained for the products of the methylation of G1P with diazomethane and their subsequent silylation

This sample was prepared identically from G1P as all the others mentioned in tables 3.4 and 3.5. It was unusual in that the glucose peak was small and that the large peak was unique to this sample.

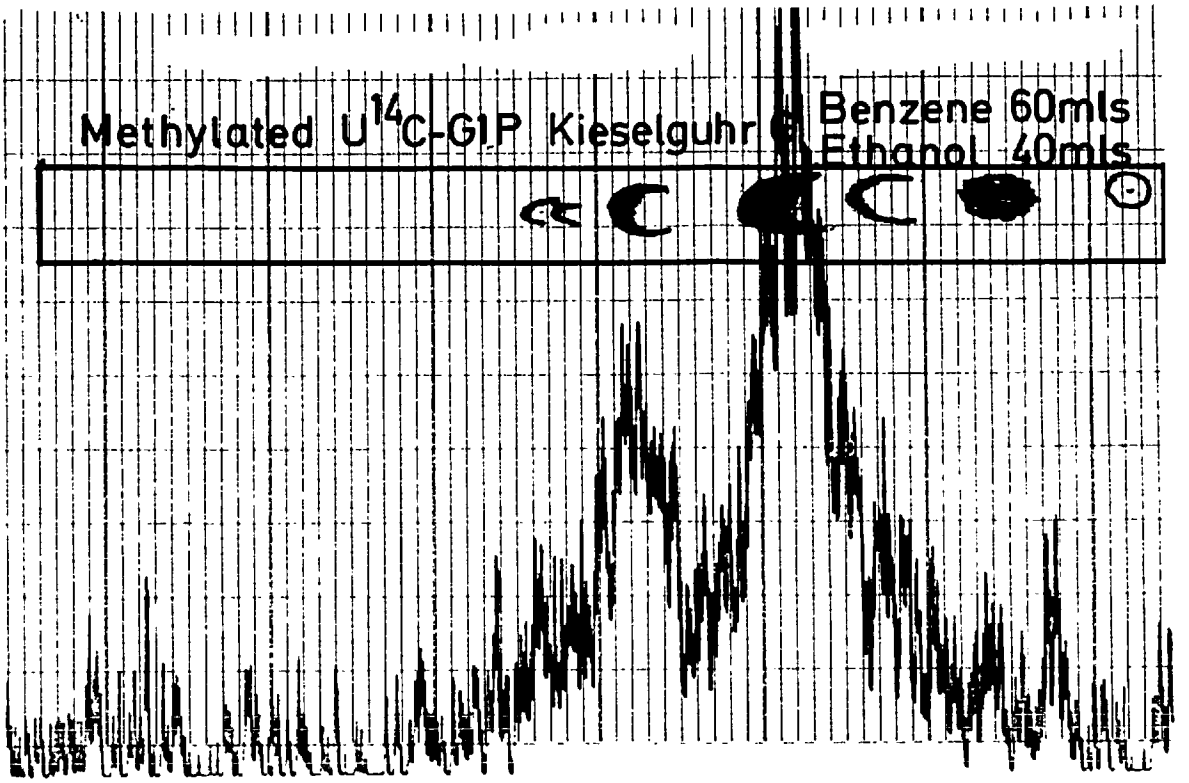

Methylated U^{14}C-GIP Kieselguhr
Benzene 60mls
Ethanol 40mls

Figure 3.8 Radioactive scanning of the TLC plate on which the separation of the products of the methylation of G1P with diazomethane had been carried out.

[U-^{14}C]-G1P was converted to the free acid in methanol with AG 50(H^+) resin. The resulting solution was reacted with diazomethane. The reaction mixture was spotted onto a 20 x 5 cm glass plate coated (250 μm thick layer) with Kieselguhr G. The plate was developed with benzene:ethanol (3:2 v/v). It was scanned for radioactivity at 12"/hour. After scanning it was sprayed with aniline-diphenylamine reagent to locate the spots. The plate was lined up with the radioactivity scan as shown opposite.

5) The form in which phosphate occurs after methylation of G1P with diazomethane

Since methylation of glucose-1-phosphoric acid in methanol led to the formation of either glucose or α-methylglucoside it was of interest to find out in what form the phosphate was in the reaction mixture.

The likely product of this reaction would be trimethyl phosphate. If phosphate were released extra diazomethane would be used to convert it to trimethyl phosphate. Indeed when higher alcohols such as isopropanol or sec-butanol were used in place of methanol, smaller amounts of ethereal diazomethane were necessary to give a permanent yellow colour than reaction with the same amount of G1P in methanol. The gas chromatographic analysis of the silylated products of the methylation gave chromatograms similar to that in Figure 3.7. However, TLC of the methylation products of G1P from different alcohols indicated no differences from the results obtained by using methanol as solvent.

Mass spectral analysis was used to look for the presence of phosphate in the reaction mixture using methanol as solvent. Firstly potassium dihydrogen phosphate was treated with AG 50 resin (H^+ form) in methanol to form the free acid. The resulting methanol solution was exhaustively treated with freshly prepared ethereal diazomethane, and then evaporated down to about 0.5 ml at 30°C under a mild vacuum. This solution was analysed by courtesy of Dr. Waight in the Department of Chemistry, Imperial College, using an MS-9 mass spectrometer. The mass spectra obtained is shown in Figure 3.9 along with data from the work of Bafus et al (1966).

The reaction mixture of G1P in methanol with diazomethane was purified by TLC on cellulose using Solvent B (see Methods). The top three cm of the plate were scraped off and eluted with methanol. The plate was not dried using heat to prevent loss of trimethyl phosphate. The methanol extract was carefully concentrated at 10°C under vacuum. This concentrate was analysed using the MS-9 mass spectrometer (see Figure 3.9).

The data obtained was not conclusive. Dr. Waight is of the opinion that trimethyl phosphate is present in the TLC extract. Certainly all the peaks measured for the methylated phosphoric acid are present in the mass spectra of the TLC extract; however many others are also present. More importantly the ratio between peak height for the peaks at m/e ratios of 27, 30, 32, 45, 47, 65, 79, 80, 127 and 140, see Table 3.6, showed no correlation.

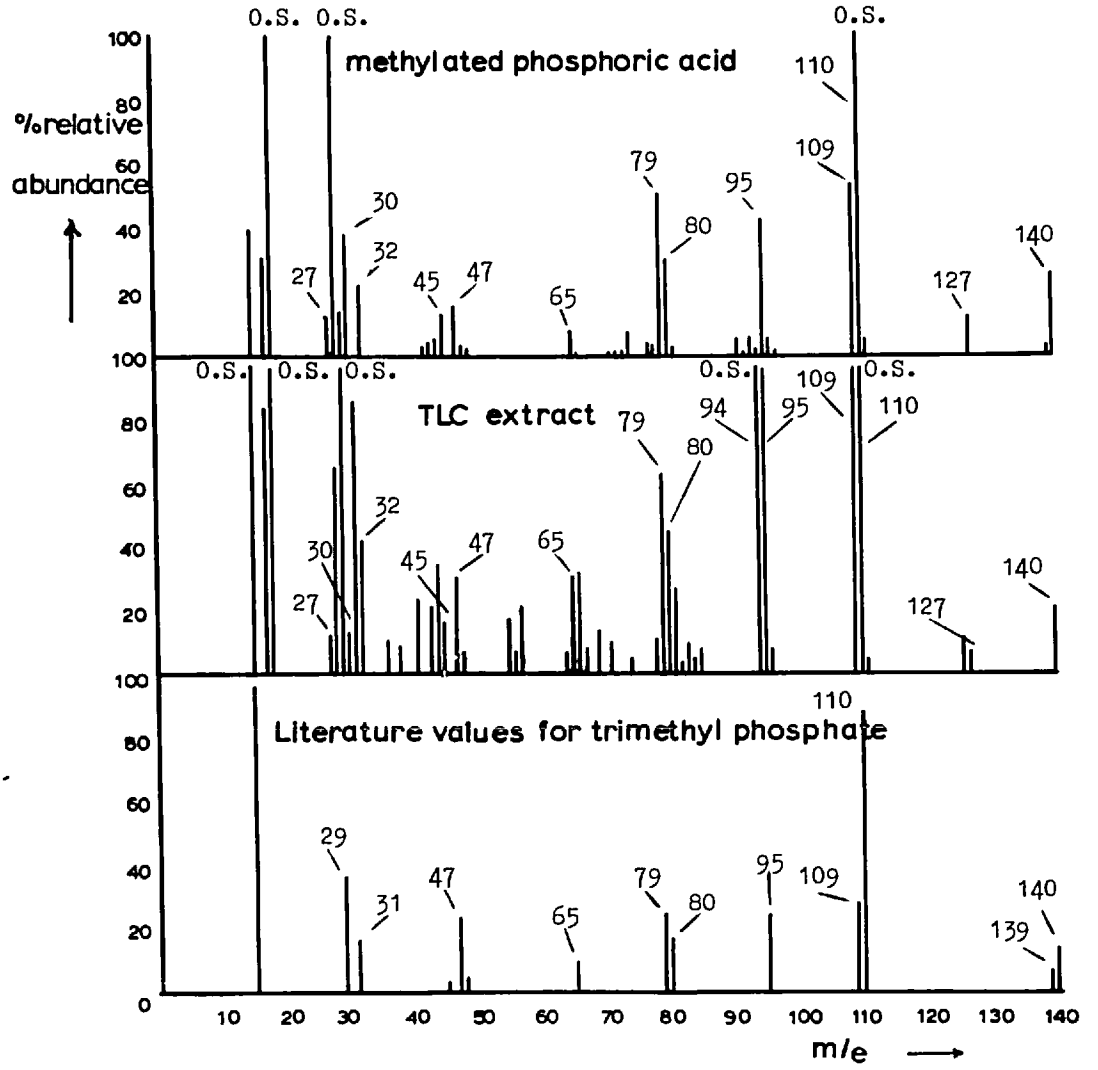

methylated phosphoric acid
TLC extract
Literature values for trimethyl phosphate
%relative abundance
O.S.
m/e
10 20 30 40 50 60 70 80 90 100 110 120 130 140

Figure 3.9 Mass spectral data used to demonstrate the presence of trimethyl phosphate in the reaction mixture of G1P and diazomethane.

The mass spectra opposite were obtained using the MS-9 mass spectrometer. Orthophosphoric acid was reacted exhaustively with diazomethane. The reaction mixture was concentrated under reduced pressure. The TLC extract was a methanol extraction of the top three cms of TLC plate (cellulose) on which a preparative separation of the products of the reaction between G1P and diazomethane had been carried out (solvent B, see Methods). No spray reagent was used before removal of this fraction. The methanol extract was concentrated carefully under reduced pressure. The literature values for the mass spectrum of trimethyl phosphate were those reported by Bafus et al (1966).

Table 3.6

Comparison of the Heights of Mass Spectral Lines in Mass Spectra reported in Figure 3.9

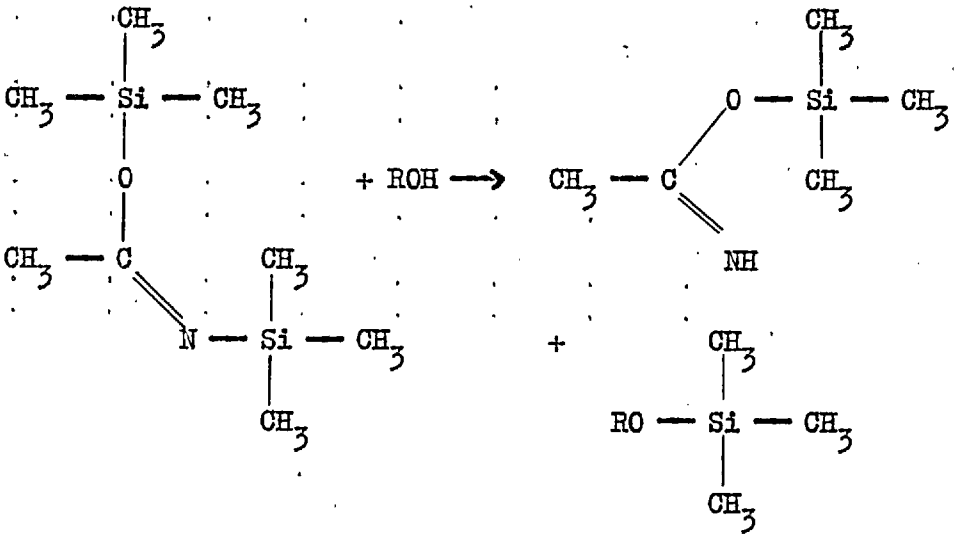
The lines corresponding to m/e ratios of 27, 30, 32, 45, 47, 65, 79, 80, 127 and 140 were compared one against another in the mass spectra for trimethyl phosphate (prepared by methylating phosphoric acid) and the TLC extract (see Figure 3.9). Each value in the table is the ratio of the heights of line m/e ratio in the row across the table to the heights of the m/e ratio in the column down the table. For each pair of data, the higher one is for the methylated phosphoric acid and the lower one for the TLC extract.

m/e values	27	30	32	45	47	65	79	80	127	140
27	- -	2.92 1.08	1.69 3.50	1.00 1.33	1.38 2.58	2.31 0.75	3.92 5.33	2.31 3.75	0.92 0.67	1.92 1.75
30	- -	- -	0.58 3.23	0.34 1.23	0.47 2.38	0.79 0.69	1.34 4.92	0.79 3.46	0.32 0.62	0.66 1.62
32	- -	- -	- -	0.59 0.38	0.84 0.74	1.36 0.22	2.32 1.50	1.36 1.07	0.55 0.19	1.24 0.50
45	- -	- -	- -	- -	1.38 1.94	2.31 0.44	3.92 4.00	2.31 2.81	0.92 0.50	1.92 1.31
47	- -	- -	- -	- -	- -	1.67 3.44	2.83 2.06	1.67 1.49	0.67 0.26	1.39 0.68
65	- -	- -	- -	- -	- -	- -	1.70 7.11	1.00 5.00	0.40 0.89	0.83 2.33
79	- -	- -	- -	- -	- -	- -	- -	0.59 0.70	0.24 0.13	0.49 0.33
80	- -	- -	- -	- -	- -	- -	- -	- -	0.40 0.18	0.83 0.47
127	- -	- -	- -	- -	- -	- -	- -	- -	- -	2.08 2.63

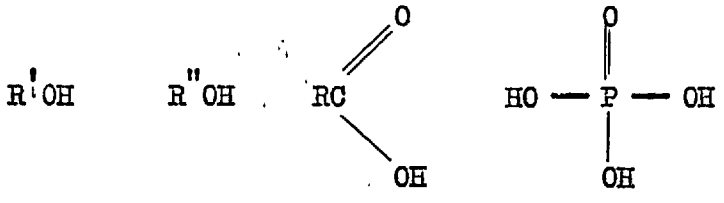
B. The trimethylsilylester, trimethylsilyl ether derivatives of sugar phosphates

1) Preparation of the derivatives

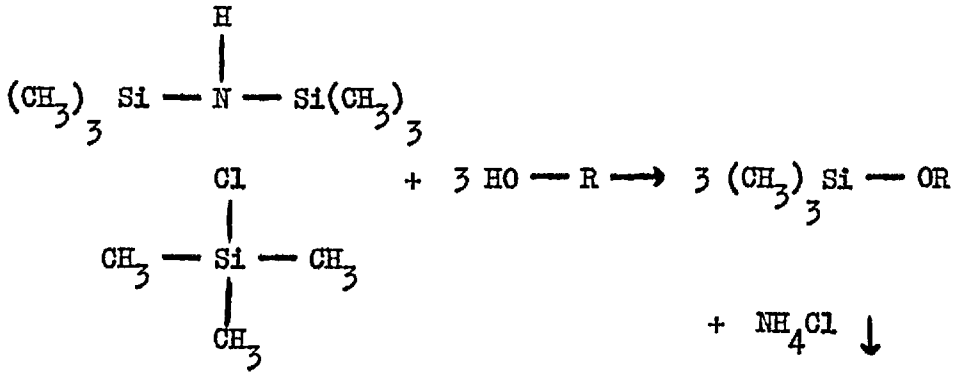
Although the derivatives of G6P and F6P could be prepared by the method of Hashizume and Sasaki (1966), a more satisfactory procedure was looked for that did not include heating under reflux. A more powerful silylating reagent than the HMDS/TMCS system was described by Klebé (1964), bis-(trimethylsilyl)-acetamide (BSA). This compound reacted with -OH groups thus:-



R = alkyl , aryl , alkanoyl , phosphate



It had the advantage that there was no precipitation during silylation as occurred with HMDS/TMCS/pyridine silylations.



The free acids of G6P, F6P, FDP and G1P were prepared from their sodium salts by passage in aqueous solution through an AG 50 (H^+ form) column (12 x 0.5 cm). The eluates were lyophilised at 30°C. The dry material was reacted with 0.5 ml BSA (maintaining anhydrous conditions) at room temperature. Aliquots were analysed using 3% SE-52 on Chromosorb W (AW-DMCS treated) column packing at 200°C. A single peak was observed for F6P, and double peaks for G6P and FDP. G1P gave rise to several peaks, not surprisingly since it presumably decomposed during lyophilisation. To prevent this the ammonium salt of G1P was formed by allowing the free acid being eluted from the column to drip into cold dilute ammonium hydroxide. This was taken to dryness and reacted with BSA (0.5 ml). This time a single peak of similar retention time to that for F6P was obtained. The derivatives for G6P, F6P and FDP were co-chromatographed with this sample (see Figure 3.10). The same peaks could be obtained by converting the sugar phosphates to their triethylammonium salts and then reacting with BSA:acetonitrile (3:2 v/v). The retention times relative to TMS-F6P are given in Table 3.7. The lability of the TMS-G1P compound was shown by the addition of TMCS to the reaction mixture. TMCS reacts with -OH groups to give HCl. As a result the peak almost completely disappeared (see Figure 3.11abc).

Attempts were made to obtain mass spectra for each of the peaks given in Figure 3.10, but this was not possible since a Ryhage molecular separator was needed so that the heavy molecules (MWT 792 for TMS-G6P) could enter the spectrometer. Such a separator is under patent and can only be obtained with an LKB 9000 GLC-mass spectrometer. Using this machine Sherman et al (1969) have obtained mass spectra for TMS-G6P and TMS-inositol-1-phosphate.

Derivatives of some other sugar phosphates were prepared with BSA and each gave single peaks when chromatographed on SE-52 or OV-17. Their retention times relative to TMS-F6P are recorded in Table 3.8.

(contd. on p.102)

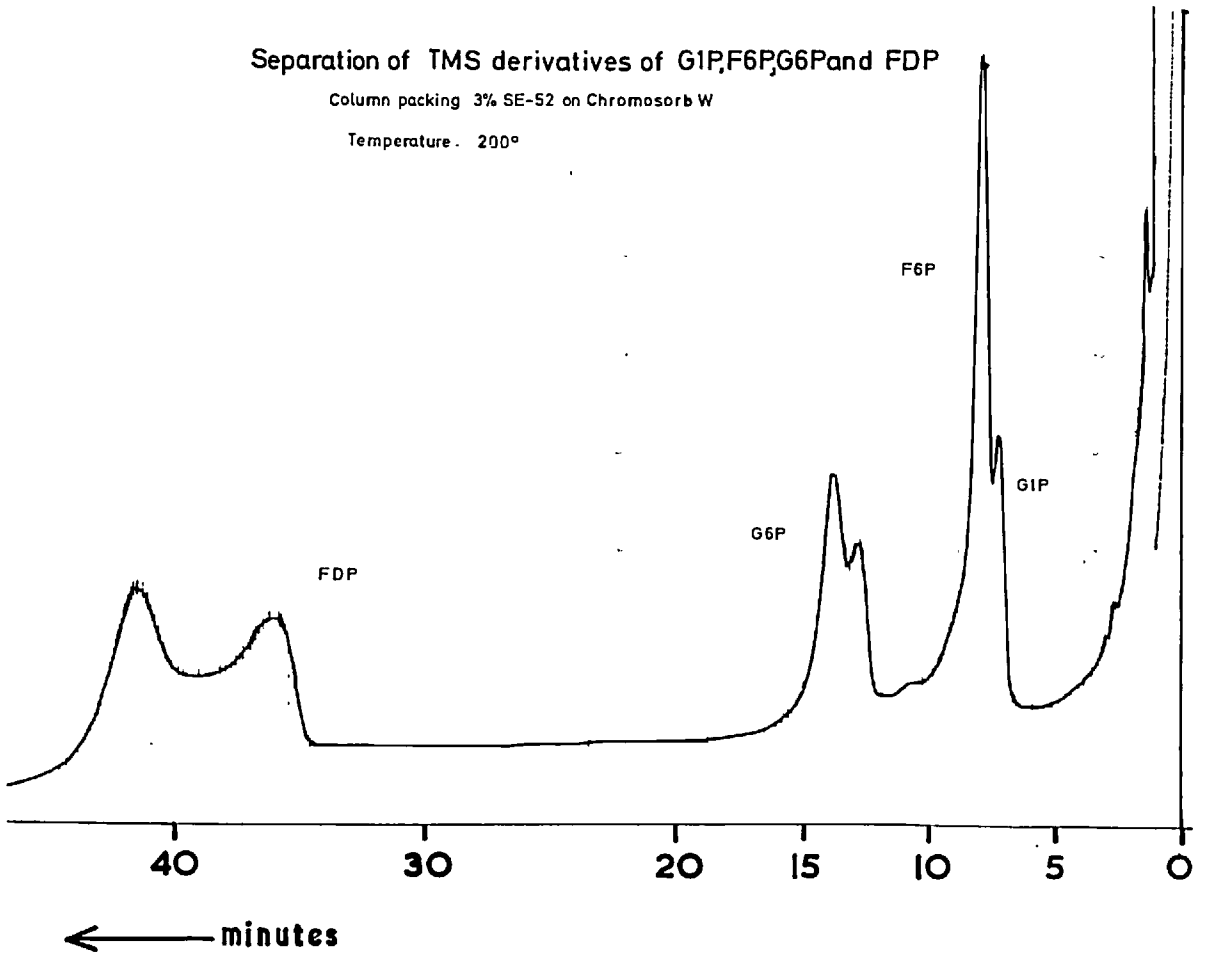

Separation of TMS derivatives of G1P,F6P,G6Pand FDP
Column packing 3% SE-52 on Chromosorb W
Temperature. 200°
F6P
G1P
G6P
FDP
40
30
20
15
10
5
0
minutes

Figure 3.10 The chromatographic separation of the trimethylsilyl ester-trimethylsilyl ether derivatives of G1P, F6P, G6P and FDP.

Each compound was reacted separately with BSA. 1 µl of each was drawn up in the same syringe and injected onto a 5' x ¼" OD glass column containing 3% SE-52 on Chromosorb W (AW-DMCS, 80-100 mesh) at 200°C and with a nitrogen flow rate of 60 ml/minute.

Table 3.7

The relative retention times of TMS ester - TMS ether sugar phosphate derivatives using 3% SE-52 on Chromosorb W

Each compound was converted to its triethylammonium salt and reacted with BSA:acetonitrile (3:2 v/v). 5 µl aliquots were analysed on 3% SE-52 on Chromosorb W (AW-DMCS, 80-100 mesh) in a 5' x $\frac{1}{4}$" column, the flow of the nitrogen carrier gas being 60 ml/min.

Compound	200°C *	220°C *
G1P	0.92	0.90
F6P	1.00	1.00
G6P	1.61, 1.74	1.58, 1.63
FDP	4.49, 5.16	4.25

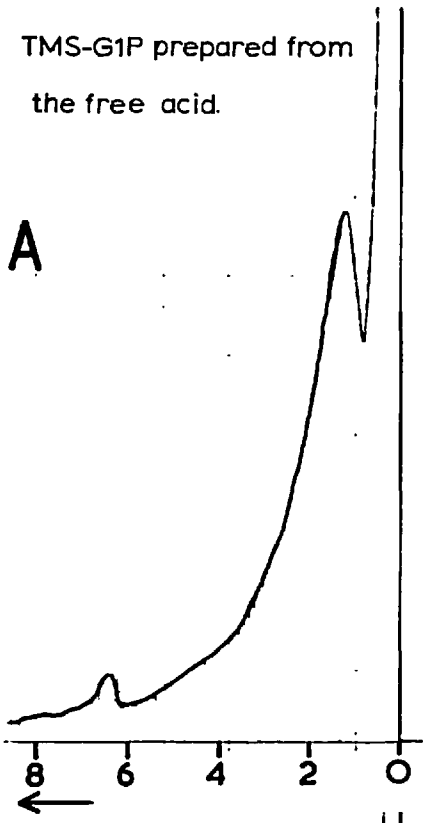
* relative to TMS-F6P, flow rate of nitrogen carrier gas 60 ml/min.

$$\text{TMS-F6P } R_T^{200^\circ C} = 7.62 \text{ minutes}$$

$$\text{TMS-F6P } R_T^{220^\circ C} = 3.15 \text{ minutes}$$

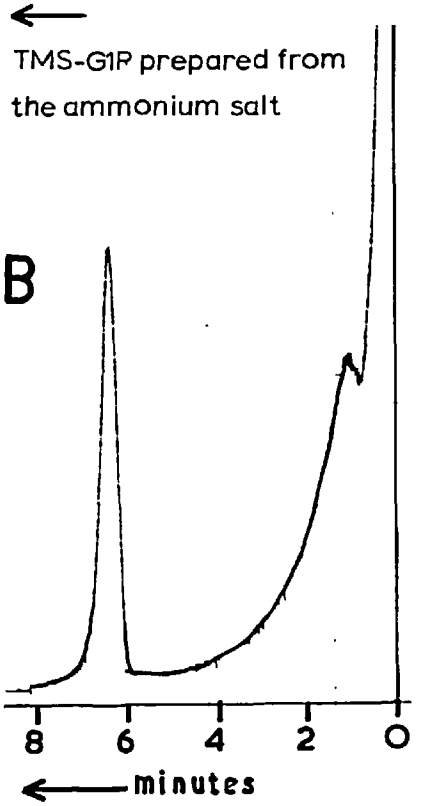
TMS-G1P prepared from the free acid.

A



TMS-G1P prepared from the ammonium salt

B



TMS-G1P after the addition of TMCS

C

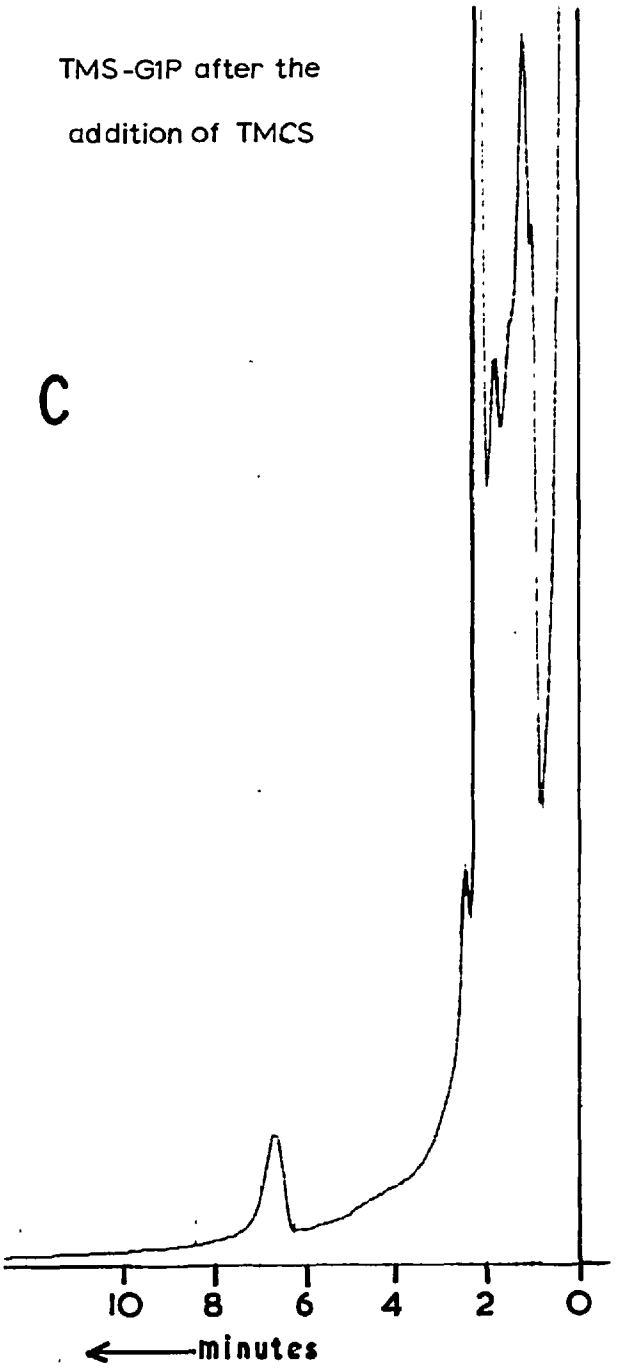


Figure 3.11 The stability of the trimethylsilyl ester - trimethylsilyl ether derivative of G1P

Figure 3.11a The free acid of G1P was formed by passage of the disodium salt through an AG 50 (H^+) resin column. It was lyophilised and reacted with BSA:acetonitrile (3:2 v/v).

Figure 3.11b Similar to Figure 3.11a except that the free acid was immediately converted to the ammonium salt before lyophilisation.

Figure 3.11c TMCS was added to the reaction mixture giving the chromatogram in Figure 3.11b. An aliquot was immediately injected.

The column packing used in each case was 3% SE-52 on Chromosorb W (AW-DMCS, 80-100 mesh). The column temperature was 200°C.

Table 3.8

The relative retention time of some TMS-sugar phosphates on 3% SE-52 and 3% OV-17

Each compound was converted to its triethylammonium salt and reacted with BSA:acetonitrile (3:2 v/v). 5 µl aliquots were analysed on 3% SE-52 or 3% OV-17 on Chromosorb W (AW-DMCS, 80-100 mesh) in a 5' x ¼" column. Flow rate of nitrogen carrier gas, 60 ml/min. Temperature of the analysis, 210°C.

Compound	3% SE-52 *	3% OV-17 +
F6P	1.00	1.00
G6P	1.66	1.84
FDP	4.25, 4.87	5.41, 6.14
6PG	- - -	1.80
Ribulose 5P	0.60	0.76
Ribose 5P	0.59	0.70
2, 3 Diphospho-glycerate	0.83	1.34
∝-Glucose	0.26	0.23

* relative to TMS-F6P on SE-52 at 210°C R_T = 5.15 minutes

\+ relative to TMS-F6P on OV-17 at 210°C R_T = 4.36 minutes

2) The quantitative analysis of sugar phosphate derivatives

It was noticed immediately that it was essential to de-activate all materials with which these derivatives came into contact. In particular the GLC column needed de-activation every day. A dozen 5 µl samples of BSA prevented a very marked depression of the peak area when first starting a series of injections. Careful drying of the molecular sieve used to dry the carrier gas, caused an increase in the response to very small samples. As a result the molecular sieve was re-activated each time when a fresh cylinder of nitrogen was necessary. Of the many column packings tried, only those with liquid phases with no active groups such as hydrocarbons and silicones could be used. In addition the support really had to be inert, Chromosorb W (AW-DMCS) or Gas Chrom Q, being suitable. The silicones were the most suitable liquid phases because of their low volatility at the temperatures needed to elute these derivatives. Methyl silicone (SE-30) was used in earliest experiments, but was rapidly superseded by methylphenyl silicone (SE-52 and OV-17).

The triethylammonium salts of the sugar phosphates, G1P, G6P, F6P and FDP dissolved immediately in BSA, an advantage when these derivatives were to be silylated in a tissue extract. Auxiliary solvents such as acetonitrile, dimethylformamide and pyridine could be used with BSA, again useful for dealing with tissue extracts if unwanted contaminants were present.

The reaction of the triethylammonium salts of G6P, F6P and FDP with BSA:acetonitrile (3:2 v/v) was reproducible (see Table 3.9). In this experiment the samples were stored overnight at 4°C. To see if

temperature affected the reaction six aliquots of a mixture of G6P, F6P and FDP were silylated with BSA:acetonitrile (3:2 v/v). Three samples were stored overnight at 4°C and three at room temperature (20-25°C). The following day gas chromatographic analysis on each was carried out. No differences could be seen for G6P and F6P, but FDP was not silylated at room temperature (see Table 3.10). Addition of more BSA did give rise to small peaks for FDP, so it would appear that a large excess of BSA was needed for FDP, some losses of BSA having occurred in the warm.

3) The recovery of trimethylsilyl derivatives from the chromatographic column

Since adsorption by the column had shown itself to be a problem in obtaining reproducible results when the column was first used each day, a check was made to ensure that all the derivative was eluted under optimum conditions. Jansen and Baglan (1968) had reported poor recoveries of some TMS sugar derivatives from silicone columns.

This was tested using the Katharometer unit as mass detector so that the whole peak could be collected without decomposition. Early results with TMS-[U-^{14}C]-glucose, trapping the peak with the scintillation cell, indicated no losses during chromatography. Similar results were obtained by merely bubbling the column effluent throught 10 ml toluene scintillant in a vial. The recoveries for TMS-[U-^{14}C]-glucose, TMS-[U-^{14}C]-fructose and TMS-[1,5-$^{14}C_2$]-citric acid are summarised in Table 3.11. As can be seen the adsorption was low, the figure of 80% for TMS-[U-^{14}C]-fructose was due to its not being purified before use.

(contd. on p.106)

Table 3.9

Reproducibility of peak heights of TMS-sugar phosphates in a mixture (separate samples)

A mixture of G6P, F6P and FDP was dissolved in water and divided into three. Each sample was converted to the triethylammonium salts (as described earlier) and reacted with BSA:acetonitrile (3:2 v/v) overnight at 4°C.

Chromatography was carried out using 3% SE-52 on Chromosorb W (AW-DMCS, 80-100 mesh) at 200°C.

P-terphenyl was used to correct for errors in injection and for adsorption by the column.

Sample	Peak ht. G6P	Peak ht. F6P	Peak ht. FDP	Peak ht. added p-terphenyl
1	21.5	9.7	2.8	13.3
2	21.7	10.1	2.9	13.7
3	21.5	9.4	3.0	13.6

Table 3.10

Effect of temperature on the silylation process

A mixture of G6P, F6P and FDP was made up in solution and divided into six equal parts. Each was converted to the triethylammonium salts, lyophilised and reacted with 0.5 ml BSA:acetonitrile (3:2 v/v). Three were stored at 4°C and three at room temperature overnight. Chromatography was carried out the following day at 200°C on 3% SE-52 on Chromosorb W (AW-DMCS, 80-100 mesh). As for Table 3.9 peak heights were used to estimate the amount of each peak.

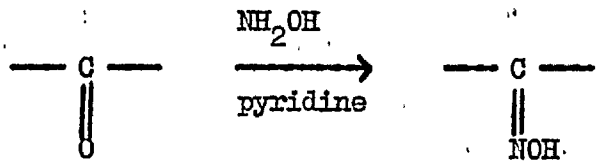
Sample	G6P		F6P		FDP	
	4°C	room temp.	4°C	room temp.	4°C	room temp.
1	15.9, 16.1	15.1, 16.2	4.6, 5.1	4.7, 5.5	7.6, 7.5	- - -
2	15.8, 15.7	17.7, 16.1	4.7, 5.2	5.9, 5.5	7.3, 7.4	- - -
3	16.8, 16.4	16.2, 16.1	5.6, 5.8	5.2, 5.6	7.5, 7.2	- - -
Mean ± S.E.M.	av.16.1 ± 0.1 (6)	16.2 ± 0.3 (6)	5.1 ± 0.2 (6)	5.4 ± 0.2 (6)	7.4 ± 0.1 (6)	

S.E.M. = standard error of mean

The sugar phosphate derivatives were tested using the flame ionisation detector with splitter (1:1) (for fuller description see Methods). Data is presented for TMS-[U-^{14}C]-G6P and TMS-[U-^{14}C]-F6P in Table 3.12. TMS-[U-^{14}C]-FDP did not give a rise of activity which coincided with the peaks attributed to it; instead activity was eluted continuously from the point of injection. G1P was not investigated since the reagent used, containing trimethylchlorosilane, would have decomposed it.

C. The trimethylsilyl derivatives of Krebs cycle intermediates

Using the method of Horii et al. (1965) derivatives were successfully prepared for lactate, pyruvate, succinate, fumarate, malate, oxaloacetate, ∝-ketoglutarate, isocitrate, and citrate. Their method involved first converting the ∝-keto groups to oximes with hydroxylamine.



This prevented side reactions occurring such as have been reported for the attempted methylations of pyruvate and ∝-ketoglutarate (Kuksis & Prioreschi, 1967). Derivatives for lactate, succinate, fumarate, malate, isocitrate and citrate were prepared using BSA:TMCS: pyridine (2:1:1 by vol). The ∝-keto acids could not be converted to a volatile form with this reagent. The method used by Horii et al (1965) would not be suitable for tissue analyses of keto-acids because it would be necessary to have a large amount of silylation reagent for the excess hydroxylamine. A better idea would be to use methoxylamine

(contd. on p.109)

Table 3.11

The percentage recovery of ^{14}C-labelled TMS-derivatives after chromatography

The figures for the Popjak cell were obtained by two procedures. 5 µl of material under test was injected down the column, condensed and counted by the scintillation equipment connected to the GLC apparatus. This was compared with the value obtained by injecting 5 µl directly into the scintillation cell. The radioactive scintillation fluids were also counted in vials in the Beckman Scintillation Spectrometer and the results compared. The figures for cold toluene were obtained by collecting the eluted vapours in 10 ml toluene cooled in ice so that there was minimal loss of toluene.

The figures for hot toluene were obtained by collecting the eluted vapours in 10 ml toluene not kept cool, up to 70% of the toluene being lost by evaporation. Toluene was added to the vial to make the volume up to 10 ml before counting.

Compound	Popjak cell	Cold toluene	Hot toluene
TMS-[U-^{14}C]-glucose	97.0 ± 3.0 (3)	- - - -	- - - -
TMS-[U-^{14}C]-fructose	80.0 ± 1.1 (6)	73.1 ± 2.5 (5)	79.6 ± 2.1 (4)
TMS-[1,5-$^{14}C_2$]-citric acid	90.3 ± 2.0 (3)	78.4 ± 1.9 (4)	74.0 ± 6.7 (4)

Means ± standard error of mean

Table 3.12

The percentage recovery of TMS-^{14}C-labelled compounds from the chromatographic column

Each of the compounds below (1 μmole, 1 μC$_i$) was passed through an AG 50 (H^+) resin column (12 x 0.5 cm) and treated with triethylamine. The samples were lyophilised and treated with BSTFA:pyridine:TMCS (2:1:1 by vol), 0.1 ml. Aliquots (5 μl, 50 nmoles) of each were injected onto a 5' x ¼" glass column containing 3% SE-52 on Chromosorb W (AW-DMCS, 80-100 mesh). The column was previously deactivated thoroughly by repeated injection of the silylation reagent. The column effluent was divided into two parts with a stainless steel splitter. One part went to the flame ionisation detector, and the other was passed to the scintillation cell. The correction factor to the radioactivity measured in the cell for splitting and counting efficiency was determined with [U-^{14}C]-toluene and found to be 3.64. The radioactivity which was eluted during the simultaneous appearance of the mass peaks was corrected and compared to that injected to give the recovery.

Compound	Recovery
[U-^{14}C]-G6P	92.1 ± 6.0 (4)
[U-^{14}C]-F6P	89.8 ± 8.2 (3)

mean ± standard error of mean

instead to form methoximes, the excess reagent not reacting with the silylating reagent. This method has been used for converting keto groups in steroids to their methoximes (Fales & Luukkainen, 1965). Recently some methoxylamine hydrochloride became available and methoximes were made of pyruvate, oxaloacetate and ∝-ketoglutarate by reacting their free acids in dry pyridine containing methoxylamine hydrochloride at 1 mg/ml at 40°C for 45 minutes. The methoximes were treated with HMDS:TMCS so as to give a pyridine:HMDS:TMCS ratio of 10:2:1 (by vol). Chromatography of these derivatives was carried out on 3% OV-1 on Gas Chrom Q using the program 100°C isothermal for 5 minutes, then temperature programming at 4°C per minute (see Figure 3.12). The retention times of the methoximes were much lower than for oximes prepared in a similar manner. The specimen of oxaloacetate was found to contain substantial amounts of pyruvate by this method.

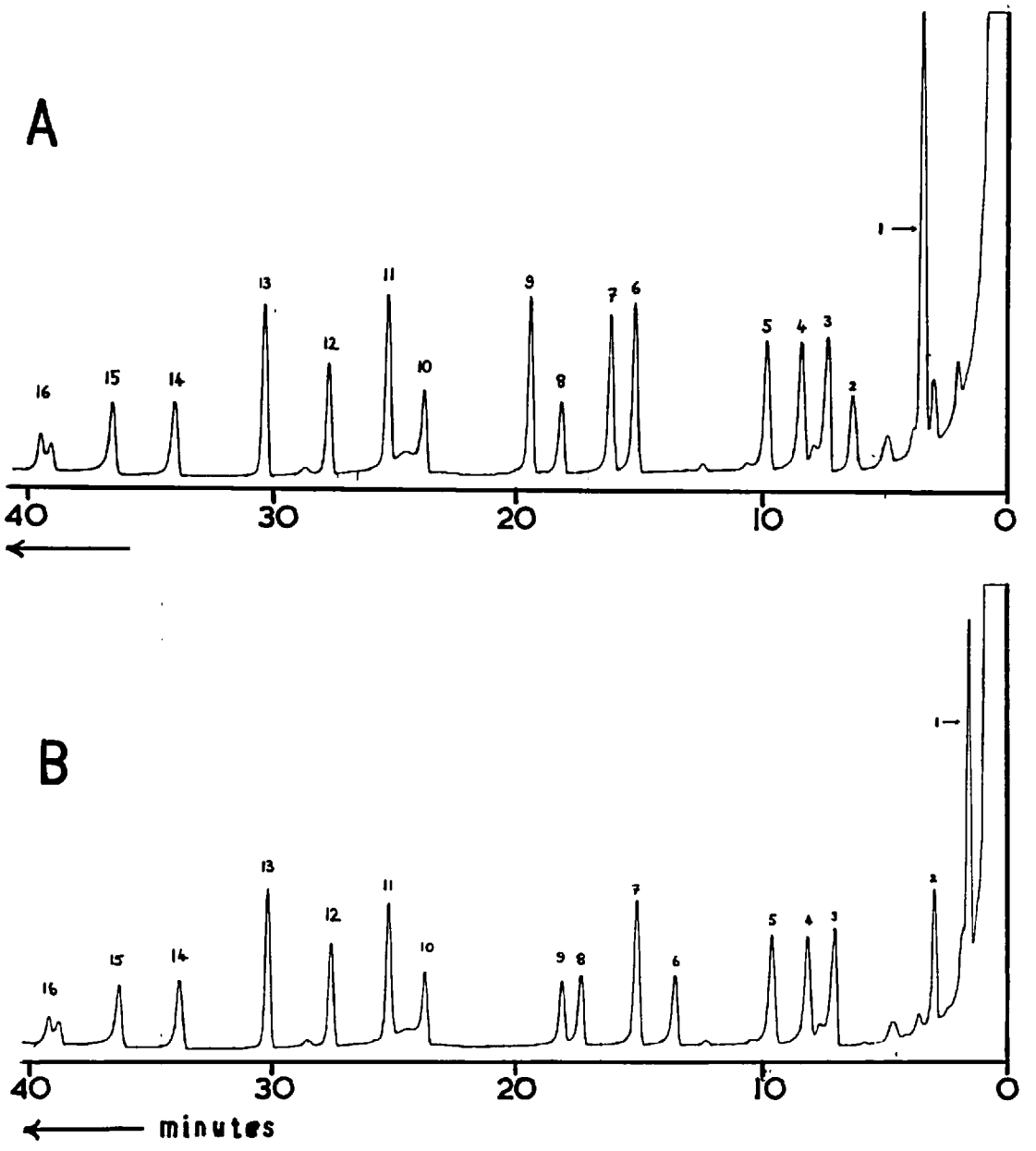

A
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minutes

Figure 3.12 The GLC separation of oximes and methoximes of pyruvate, oxaloacetate and ∝-ketoglutarate as their TMS-derivatives on 3% OV-1 on Gas Chrom Q.

The oximes were prepared from each ∝-keto acid by reaction in pyridine with hydroxylamine hydrochloride. The oximes were converted to TMS-derivatives with hexamethyldisilazane and trimethylchlorosilane. The methoximes were prepared using methoxylamine hydrochloride in a similar manner.

Gas chromatography was carried out on 3% OV-1 on Gas Chrom Q using the temperature programme: 100°C for 5 minutes, then linear temperature programming 100-250°C at 4°C per minute.

A. Separation of the oximes

Peak identification: 1. pyruvate 2. unknown impurity 3. phosphate 4. succinate 5. fumarate 6. malate 7. oxaloacetate 8. hexadecane 9. ∝-ketoglutarate 10. ∝-glycero-phosphate 11. citrate 12. ∝-D-glucose 13. β-D-glucose 14. docosane 15. fructose-6-phosphate 16. glucose-6-phosphate

B. Separation of the methoximes

Peak identification: 1. pyruvate 2. unknown impurity 3. phosphate 4. succinate 5. fumarate 6. oxaloacetate 7. malate 8. ∝-ketoglutarate 9. hexadecane 10. ∝-glycerophosphate 11. citrate 12. ∝-D-glucose 13. β-D-glucose 14. docosane 15. fructose-6-phosphate 16. glucose-6-phosphate

CHAPTER 4

Development of a suitable method for GLC analysis of the intermediates being studied

In the previous chapter it was demonstrated that TMS-derivatives of both sugar phosphates and organic acids could be prepared relatively simply, and that there were conditions for separating them. In this chapter investigation of temperature programmed analysis was carried out in order to separate all of these derivatives in a single chromatographic run. This entailed the use of a new silylating reagent, bis-(trimethylsilyl)-trifluoroacetamide (BSTFA). Chromatographic data for three liquid phases was determined.

1) The development of a temperature programme

The chromatographic conditions used to separate TMS-G1P, TMS-G6P and TMS-F6P mentioned in the previous chapter (isothermal analysis at 200-220°C) were not suitable for the more volatile TMS-derivatives such as lactate, fumarate, succinate and malate. Operation at lower temperature would have increased the retention times of TMS-G6P and TMS-F6P to impractical times, and also spread out the peaks such that they would be difficult to distinguish from the standing current. Temperature programmed analysis has the advantage that a wide range of volatile substances can be examined in a reasonable length of time, and since the steadily increasing temperature sharpens the peaks, their width does not increase with increased retention time.

The starting point of the programmes used was 100°C (a few analyses were started at 110°C). This was higher than in the programme

of Horii et al (1965) but still enabled lactate to be separated from the solvent front. The programming rate was decided by examining the chromatography of an extract of liver (for details of isolation see Chapter 5). The difference between 2°C and 4°C per minute is shown in Figure 4.1. For the work with radioactive measurements there had to be a sufficient separation between individual peaks to enable the count rate in the scintillation cell to be measured. This was not possible in every case (see fumarate and succinate). The rate of 2°C per minute was about the slowest rate which was compatible with a reasonable length of analysis for radioactive compounds. When only mass measurements were to be made a higher rate of 4°C per minute was possible.

The last compound to be eluted, TMS-G6P, came off the columns used at 230-240°C so the programme was continued to 250°C. When radioactive extracts were being studied if the programme was terminated below 250°C a steady accumulation of radioactivity occurred in the scintillation cell at the start of the next run.

The temperature programme 100-250°C at 2°C per minute was used with the column packings 3% SE-52 and 3% OV-17 on Chromosorb W (AW-DMCS, 80-100 mesh). Specimen chromatograms are shown in Figure 4.2. Retention times of many intermediates are given in Table 4.1.

The most recent studies have been carried out using the column packing 3% OV-1 on Gas Chrom Q (100-120 mesh) and the separations obtained are shown in Figure 4.3. Since radioactivity measurements were not made the temperature programme : 100°C for 5 minutes, then 100-250°C at 4°C per minute was used. This cut the analysis time down from 65 minutes to 42 minutes.

2) Problems associated with temperature programming

The work carried out with the liquid phases SE-52 and OV-17 used a dual column analysis, the second column being a blank, to compensate for the marked increase in the bleeding of these liquid phases with increased temperature. With OV-1 as the liquid phase, once the column had been heat treated at 260°C for 48 hours, there was very little baseline drift and a single column analysis was possible at all sensitivities used (down to attenuation X100).

In the dual column studies using the silylation reagent BSA: TMCS:pyridine (2:1:1, by vol) a hump appeared in the chromatograms from 180-230°C (see Figure 4.1). Elimination of other possibilities showed that the BSA was responsible. Following a suggestion of Horning et al (1967) the stationary phase was additionally silylated with BSA and TMCS dissolved in toluene before being coated with SE-52 liquid phase. This had no effect on the hump however.

Gehrke et al (1968) used a derivative of BSA, bis(trimethylsilyl) -trifluoroacetamide, for the silylation of amino acids. They found it to be a superior silylating reagent, being a more powerful silyl donor, and also that monotrimethylsilyl trifluoroacetamide was very much more volatile than monotrimethylsilylacetamide, the latter compound being eluted some way after the solvent front, interfering with analysis of TMS-glycine.

BSTFA was used in place of BSA in this study and the hump was almost eliminated. Also it was noticed that the tailing and extent of the solvent front was due to the pyridine when BSTFA was used. With a few exceptions all the silylations reported from here on used BSTFA instead of BSA.

(contd. on p. 122)

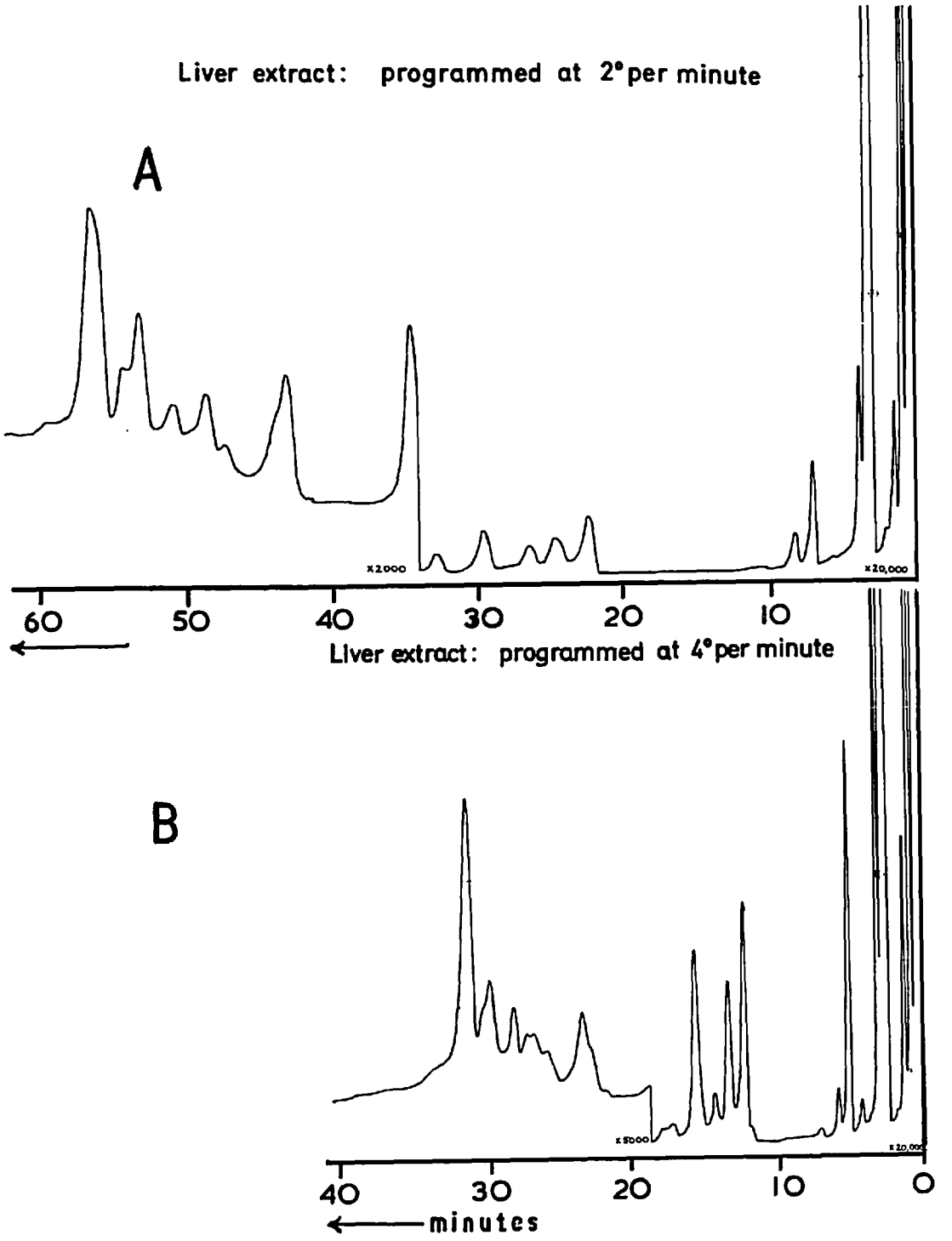

Liver extract: programmed at 2° per minute
A
x2000
x20,000
60
50
40
30
20
10
Liver extract: programmed at 4° per minute
B
x5000
x20,000
40
30
20
10
0
minutes

Figure 4.1 The effect of changing the temperature programming rate on the separation by gas-liquid chromatography of trimethylsilyl derivatives of compounds isolated from liver

Rat liver was freeze clamped *in situ*, and 5 g of frozen liver extracted twice with 60% aqueous ethanol. The extract was absorbed onto an AG 1 (formate form) resin column (12 x 0.5 cm). After washing with 10 x 10 ml water, the column was eluted with 4 x 10 ml 2M formic acid. The eluate was evaporated to dryness and then reacted with BSA: pyridine:TMCS (2:1:1 by vol) 0.1 ml. 5 µl was injected onto a 3% SE-52 on Chromosorb W (AW-DMCS, 80-100 mesh) 5' x ¼" column. The temperature programmes used were 110-230°C at 2°C per minute (Figure A), and 110-230°C at 4°C per minute (Figure B).

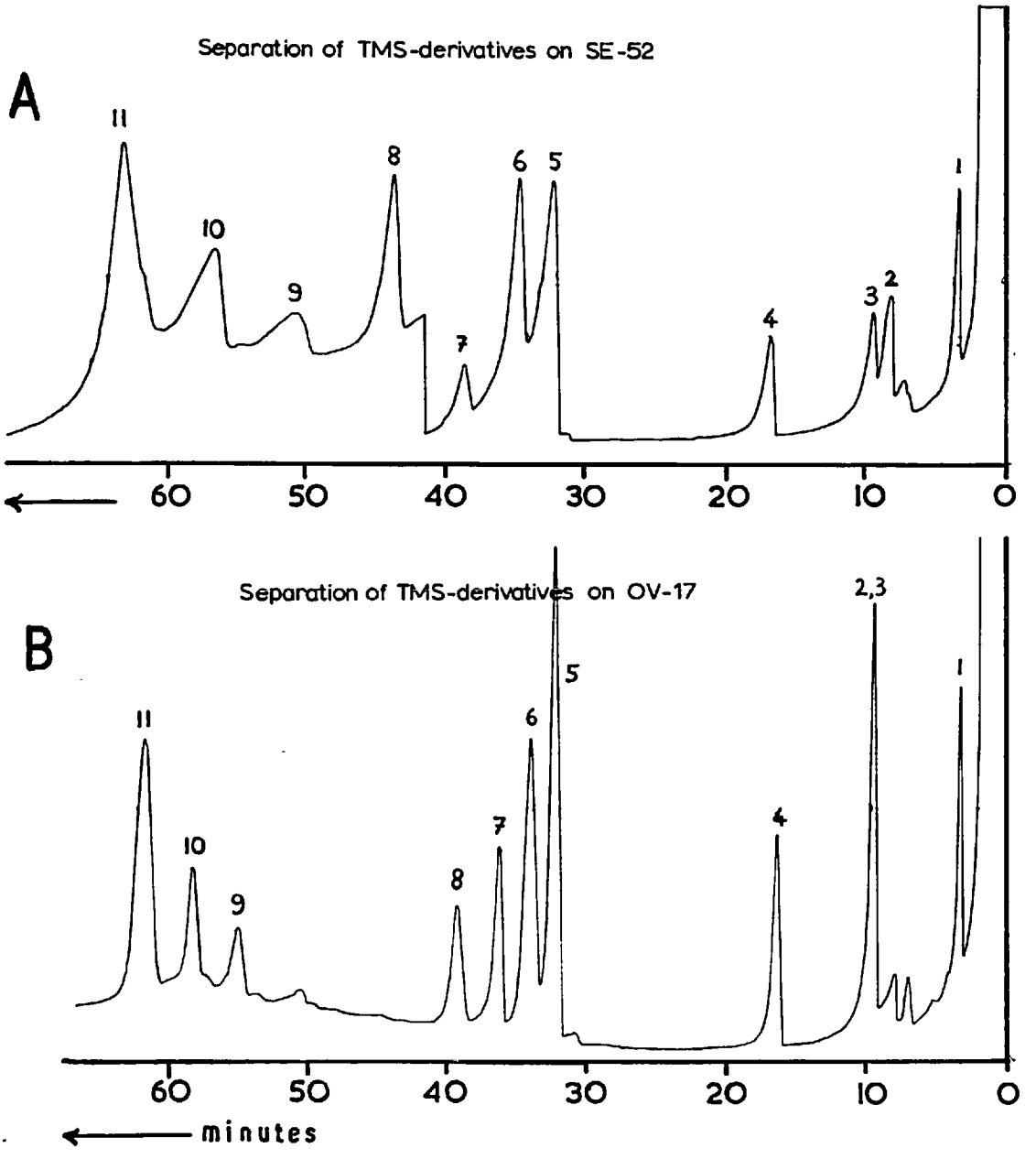

Separation of TMS-derivatives on SE-52
A
11
10
9
8
7
6
5
4
3
2
1
60
50
40
30
20
10
0
Separation of TMS-derivatives on OV-17
B
11
10
9
8
7
6
5
4
2,3
1
60
50
40
30
20
10
0
minutes

Figure 4.2 Temperature programmed gas chromatographic analysis of some trimethylsilyl ester-trimethylsilyl ether derivatives on SE-52 and OV-17 liquid phases

A mixture of each of the compounds named was treated with 0.1 ml BSTFA:pyridine:TMCS (2:1:1, by vol) after lyophilisation. 5 µl of the reaction mixture was injected onto either 3% SE-52 or 3% OV-17 on Chromosorb W (AW-DMCS, 80-100 mesh) columns (5' x $\frac{1}{4}$") using the temperature programme 100-250°C at 2°C per minute.

Identification of peaks:

1. β-hydroxybutyrate 2. succinate 3. fumarate 4. malate
5. α-glycerophosphate 6. citrate 7. α-glucose 8. β-glucose
9. ribose-5-phosphate 10. fructose-6-phosphate
11. glucose-6-phosphate

Table 4.1

The retention times of some TMS-derivatives on SE-52 and OV-17

The TMS-derivatives of these compounds were chromatographed on 3% SE-52 and OV-17 on Chromosorb W (AW-DMCS, 80-100 mesh) using a temperature programme 100-250°C at 2°C per minute. The nitrogen carrier gas flow rate was 60 ml/minute.

Derivative	SE-52	OV-17
β-hydroxybutyrate	3.6 mins	3.3 mins
succinate	7.3	9.5*
fumarate	9.6	9.5*
malate	16.9	16.4
α-glycerophosphate	32.4	31.7
citrate	34.7	33.4
3-phosphoglyceric acid	36.4*	37.2
α-glucose	37.6	35.4
β-glucose	40.2	39.4
ribose-5-phosphate	50.6	50.6
2,3 diphosphoglycerate	- - -	58.3
fructose-6-phosphate	56.5	55.0
glucose-6-phosphate	63.0	61.6

* Not separated in mixture, the value given is that obtained in a separate assay with the pure substance.

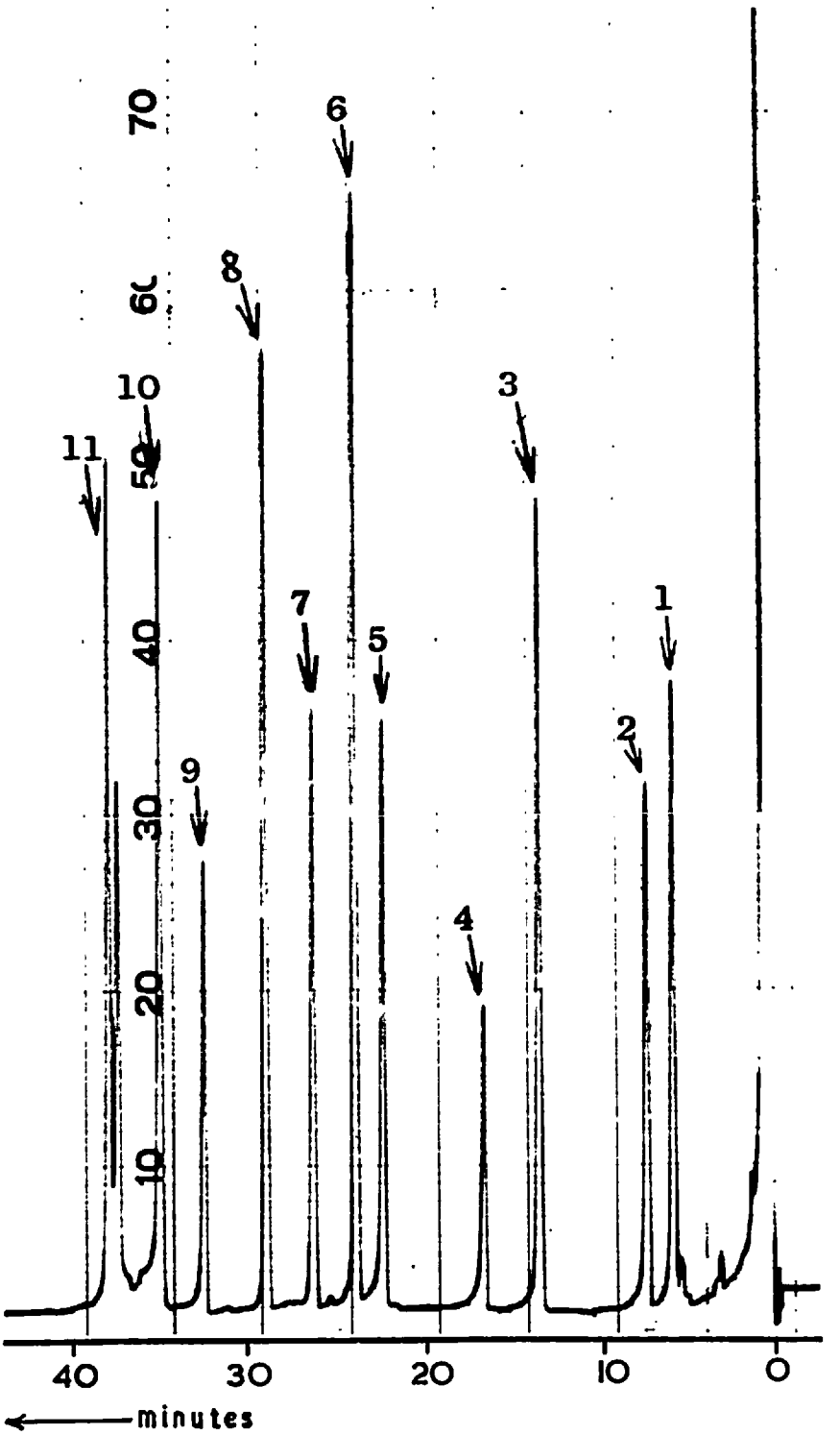

70
60
50
40
30
20
10
11
10
8
6
9
7
5
4
3
2
1
40
30
20
10
0
minutes

Figure 4.3 GLC separation of TMS-derivatives on 3% OV-1 on Gas Chrom Q

A mixture of the ammonium salts of each compound (500 nmole each) was reacted with 0.1 ml BSTFA:pyridine:TMCS (2:1:1, by vol). Gas chromatographic analysis was carried out using 3% OV-1 on Gas Chrom Q (100-120 mesh) column packing in a 5' x $\frac{1}{4}$" column. The temperature programme : 100°C for 5 minutes, then temperature programming 100-250°C at 4°C per minute was used. The temperature of the F.I.D. detector was 250°C. Nitrogen flow rate was 60 ml per minute measured at 100°C. 5 µl aliquots were injected (i.e. 25 nmoles). The attenuation setting of the amplifier was 1 x 10^4.

Peak identification:

1. succinate 2. fumarate 3. malate 4. hexadecane 5. α-glycerophosphate 6. citrate 7. α-D-glucose 8. β-D-glucose 9. docosane 10. fructose-6-phosphate 11. glucose-6-phosphate

3) Reproducibility of quantitative analyses

Relative molar responses were determined for a range of compounds (1 μmole of each present in the silylation mixture) separated on the SE-52 and OV-17 columns. The internal standards, p-terphenyl and 1, 1, 2, 2-tetraphenyl ethylene were used. p-terphenyl interfered with TMS-G6P on OV-17, and 1, 1, 2, 2-tetraphenyl ethylene with TMS-G6P on SE-52. Thus although they could be used to correct the injection aliquot for volume differences they were not suitable as reference peaks. Instead TMS-glucose (combined peaks) was used, and the relative molar responses given in Table 4.2 are relative to TMS-glucose. The responses were the same for each column.

The reproducibility and the relative molar responses of certain compounds have been studied in more detail on the column, 3% OV-1 on Gas Chrom Q (100-120 mesh), 5' x $\frac{1}{4}$". This phase was superior to those mentioned before, showing a greater efficiency (2600 plates per 5' column measured at 200°C with docosane) than SE-52 or OV-17. There was no noticeable deterioration of this efficiency over five months whereas intensive use of the 3% SE-52 on Chromosorb W (AW-DMCS 80-100 mesh) column resulted in tailing of the type shown in Figure 4.2A.

Relative molar responses have been calculated relative to TMS-glucose (for comparison with previous data) and also relative to the two standards used, the straight chain hydrocarbons, hexadecane (C16) and docosane (C22). These values were determined at three different concentrations of the compounds being studied, at 0.5 μmole, 0.2 umole and 0.04 μmole (see Tables 4.3 and 4.4). The values for the phosphate containing compounds show a decrease of the relative molar response with decreasing concentration. The organic acids and glucose gave consistent values with decreasing concentration.

(Contd. on p. 127)

Table 4.2

The relative molar response of some TMS-derivatives on 3% SE-52 and 3% OV-17 on Chromosorb W (AW-DMCS)

A mixture of the compounds was made up so that a 5 µl aliquot after silylation contained 0.05 µmoles of each. Also individual injections of 0.05 µmoles of each intermediate were carried out on 3% OV-17 and SE-52 on Chromosorb W (AW-DMCS, 80-100 mesh), 5' x ¼" columns. No difference was found for each compound between each column, or between individual injections and group injections.

Compound	RMR*
Succinate	0.56
Fumarate	0.47
Malate	0.63
Citrate	0.97
3PGA	0.63
Glucose	1.00
F6P	0.41
DPG	0.47
6PG	0.78
G6P	0.84

* relative to the response for glucose

Table 4.3

The relative molar responses of some TMS-derivatives to TMS-glucose (α and β forms combined) on 3% OV-1 on Gas Chrom Q

The test compounds including glucose were mixed in aqueous solution so that each was present at a concentration of 1 mM. 1 ml aliquots of this solution were passed through 2 x 0.5 cm columns of AG 50W (H^+ form) resin (100-200 mesh). The columns were washed with 4 x 1 ml distilled water. 1 drop of triethylamine was added to form the triethylammonium salts. From each eluate 2.5, 1.0 and 0.2 ml aliquots were removed and were evaporated to dryness in separate specimen tubes (75 x 12 mms) over phosphorous pentoxide under vacuum. The compounds were reacted with 100 µl of silylation reagent (BSTFA: pyridine:TMCS, 2:1:1 by vol) which also contained 0.16 µmole of hexadecane and docosane internal standards (see Table 4.4). The samples were stored overnight at 4°C. Gas chromatographic analysis was carried out by injecting 5 µl aliquots on a 3% OV-1 on Gas Chrom Q (100-120 mesh) 5' x $\frac{1}{4}$" column. The temperature programme used was: Isothermal 100°C for 5 minutes, then linear temperature programming at 4°C per minute, 100-250°C. Peak areas were determined by triangulation. The values presented below are the ratio of the peak area of each compound to that of the combined areas of TMS-α-D-glucose and TMS-β-D-glucose. Since the compounds were present in equimolar amounts no further correction was necessary. Results are expressed as arithmetic means ± the standard error of the mean based on 8 results.

(contd. on next page)

Table 4.3

(contd. from previous page)

Compound	Amount of Compound Reacted		
	0.5 µmole	0.2 µmole	0.04 µmole
Succinate	0.500 ± 0.029	0.504 ± 0.038	0.479 ± 0.023
Fumarate	0.456 ± 0.029	0.479 ± 0.034	0.450 ± 0.027
Malate	0.615 ± 0.036	0.652 ± 0.033	0.655 ± 0.031
∝-Glycerophosphate	0.442 ± 0.025	0.421 ± 0.030	0.225 ± 0.014
Citrate	0.775 ± 0.026	0.816 ± 0.020	0.749 ± 0.026
Fructose-6-Phosphate	0.598 ± 0.017	0.465 ± 0.031	0.315 ± 0.033
Glucose-6-Phosphate	0.934 ± 0.027	0.854 ± 0.051	0.586 ± 0.044

Table 4.4

The relative molar response of some TMS-derivatives to hexadecane and docosane internal standards on 3% OV-1 on Gas Chrom Q

The samples were treated and analysed as described in Table 4.3. The values presented below are derived from the ratio of the peak area of each compound to that of the internal standards hexadecane and docosane. Correction to give the relative molar response was carried out by multiplying this ratio by the ratio of the amount of standard present (0.16 umole) to that of the test compounds in the samples (0.5, 0.2 and 0.04 umoles). Results are expressed as arithmetic means ± the standard errors of the mean based on 8 results.

a) Hexadecane

Compound	0.5 μmole	0.2 μmole	0.04 μmole
Succinate	0.736 ± 0.044	0.674 ± 0.024	0.831 ± 0.039
Fumarate	0.648 ± 0.011	0.664 ± 0.028	0.802 ± 0.040
Malate	0.884 ± 0.015	0.895 ± 0.014	1.094 ± 0.027
∝-Glycerophosphate	0.638 ± 0.005	0.588 ± 0.014	0.395 ± 0.026
Citrate	1.136 ± 0.074	1.155 ± 0.012	1.247 ± 0.055
Glucose (∝ + β forms)	1.505 ± 0.086	1.411 ± 0.034	1.653 ± 0.036
Fructose-6-Phosphate	0.947 ± 0.059	0.681 ± 0.045	0.563 ± 0.017
Glucose-6-Phosphate	1.381 ± 0.075	1.221 ± 0.063	1.004 ± 0.046

b) Docosane

Compound	0.5 μmole	0.2 μmole	0.04 μmole
Succinate	0.566 ± 0.045	0.512 ± 0.023	0.564 ± 0.011
Fumarate	0.499 ± 0.020	0.504 ± 0.028	0.544 ± 0.022
Malate	0.682 ± 0.034	0.684 ± 0.024	0.744 ± 0.031
∝-Glycerophosphate	0.490 ± 0.024	0.452 ± 0.025	0.202 ± 0.020
Citrate	0.874 ± 0.055	0.880 ± 0.033	0.864 ± 0.033
Glucose (∝+β forms)	1.098 ± 0.075	1.076 ± 0.039	1.144 ± 0.025
Fructose-6-Phosphate	0.685 ± 0.038	0.524 ± 0.040	0.392 ± 0.010
Glucose-6-Phosphate	1.062 ± 0.052	0.932 ± 0.059	0.664 ± 0.055

By plotting the product of the relative molar response and the amount of each compound present against the amount of each compound present a mass response curve was obtained (see Figure 4.4). As may be seen, the curve for each compound was essentially linear, even for the phosphate containing compounds. The lines for the phosphate compounds however did not pass through the origin when extrapolated to the 'x' axis. The intercept in each case was about 20 nmoles. This would mean that for each injection onto the GLC column used about 1 nmole would be irreversibly bound to the column.

To examine this curve in greater detail a standard mixture of the test compounds was made up, and aliquots corresponding to 25, 50, 100, 200, 300, 400 and 500 nmoles taken and silylated. GLC was carried out on the 3% OV-1 on Gas Chrom Q column. The relative area response to a single concentration of docosane (internal standard) was plotted against the concentration of the test compounds (see Figure 4.5). As can be seen, the lines were indeed straight as suspected above. Best straight line calculations showed that G6P and F6P had large negative values for the constant in the equation $y = mx + c$ (Table 4.5). α-glycerophosphate however had only a small constant, because of the curved nature of the lower part of its response curve.

In addition at each concentration the amount of the standards added was also varied to see whether a linear relationship held between each substance and the standard (see Table 4.6). Docosane gave a reasonably close correlation to the added ratio of 10:2:1. Hexadecane was not so good, there being a deviation at the lowest amount added (0.016 μmoles).

Taking the chromatograms used to obtain the data in Tables 4.2, 4.3, 4.4 the correlation between peak area and peak height was

examined. As can be seen in Table 4.7 this correlation was good, the percentage standard error of the mean did not exceed 2.5% and was mainly around 1%.

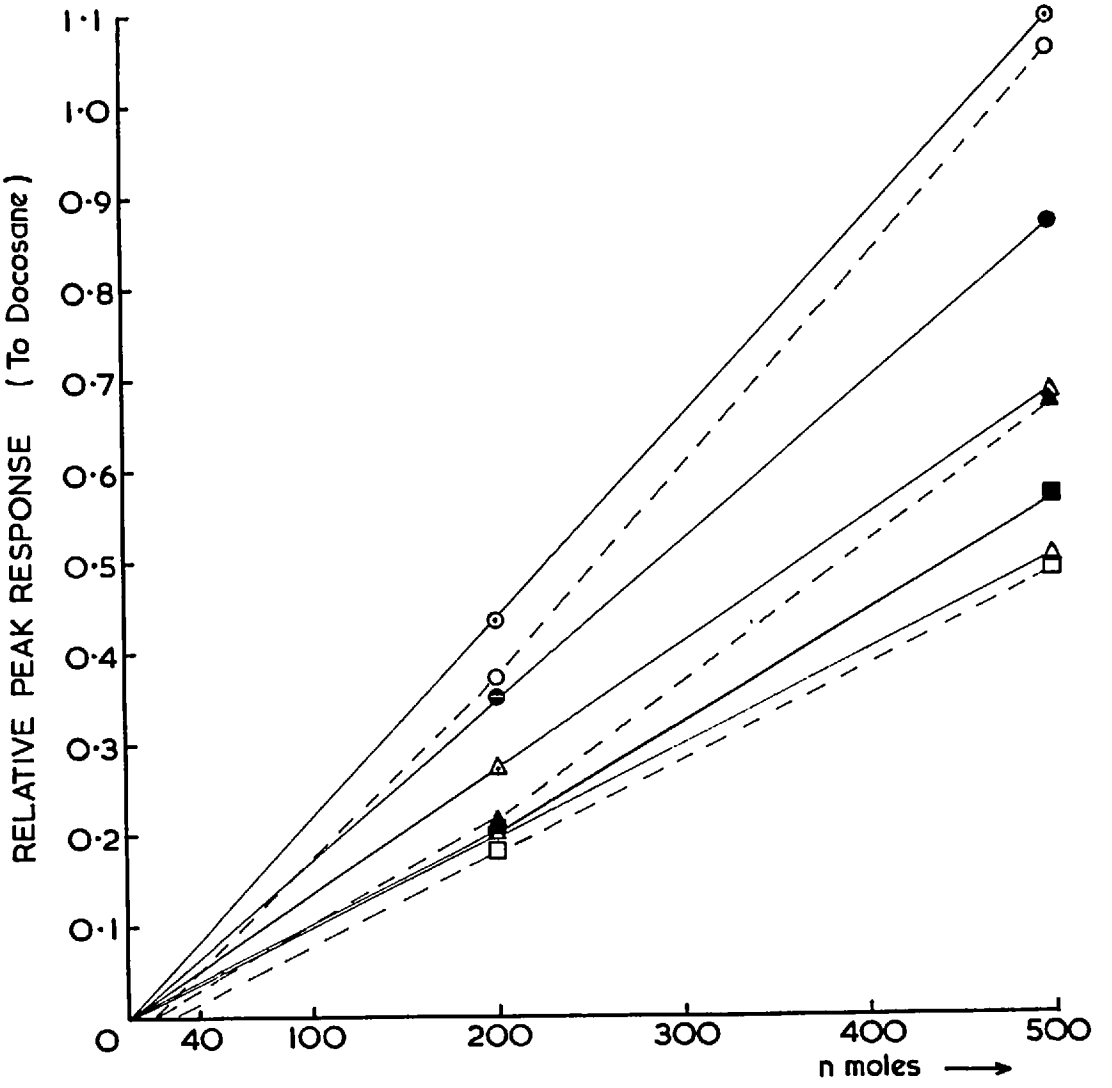

RELATIVE PEAK RESPONSE (To Docosane)
1·1
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0
40
100
200
300
400
500
n moles

Figure 4.4 The area response versus concentration curve calculated from the relative molar response at each concentration

The compounds in the diagram were made up in three mixtures containing 40, 200 and 500 nmoles of each compound as its triethyl-ammonium salt. Each was reacted with 0.1 ml BSTFA:pyridine:TMCS (2:1:1 by vol) containing 160 nmoles of docosane. Analysis was carried out as described in Tables 4.3 and 4.4. The relative molar responses at each concentration so calculated were multiplied by the amount of each compound. This was the area response with respect to a single amount of docosane. It was then plotted against the amount of each compound.

Identification:

- ⊙ glucose
- ○ glucose-6-phosphate
- ● citrate
- △ malate
- ▲ fructose-6-phosphate
- ■ succinate
- △ fumarate
- □ -glycerophosphate

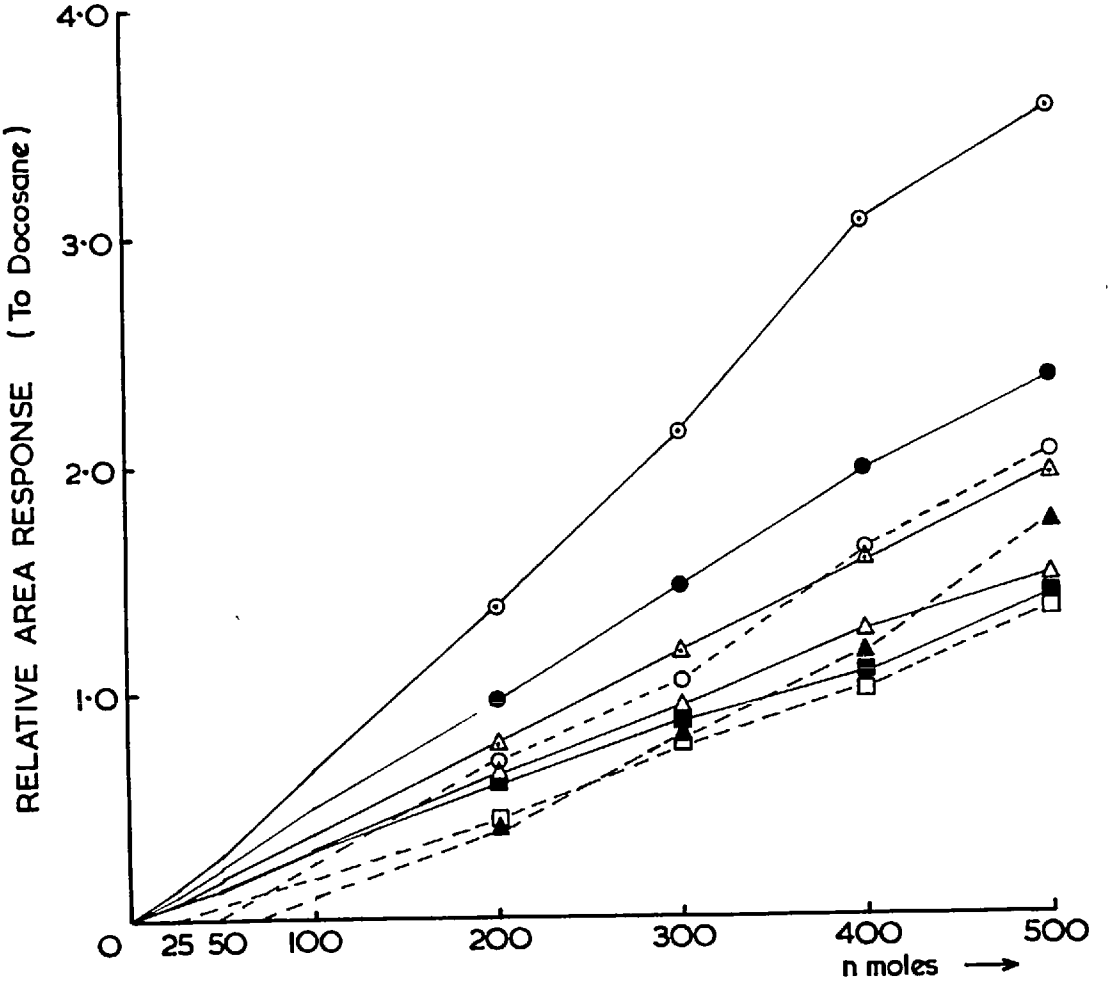

RELATIVE AREA RESPONSE (To Docosane)
4·0
3·0
2·0
1·0
0
25
50
100
200
300
400
500
n moles

Figure 4.5 The area response relative to a single amount of docosane versus the amount of substance present

The compounds in the diagram were made in mixtures containing the following amounts, 25, 50, 100, 200, 300, 400 and 500 nmoles, as their triethylammonium salts. Each was silylated with 0.1 ml BSTFA: pyridine:TMCS (2:1:1 by vol) containing 80 nmole docosane. Gas chromatographic analysis was carried out on 3% OV-1 on Gas Chrom Q (100-120 mesh) column packing in a 5' x ¼" column using the temperature programme : 100°C for 5 minutes, then temperature programming 100-250°C at 4°C per minute. Peak areas for each compound were divided by the area of the standard to give the relative area response. This was plotted against the total amount of each compound in the silylation reaction mixture.

Identification:

- ⊙ glucose
- ◉ citrate
- ○ glucose-6-phosphate
- ◬ malate
- ▲ fructose-6-phosphate
- △ fumarate
- ■ succinate
- □ α-glycerophosphate

Table 4.5

Statistical analysis for the best straight line

25, 50, 100, 200, 300, 400 and 500 nmoles of each of the compounds below were silylated, with docosane as internal standard, and analysed by GLC on 3% OV-1 on Gas Chrom Q (100-120 mesh), 5' x $\frac{1}{4}$" column. The relative area response to docosane was plotted against the amount of each compound present (see Figure 4.5).

The best straight line for the response curve for each compound was calculated statistically, as $y = mx + c$. x was the amount of compound in nanomoles.

Compound	Values of m	Values of c
∝-glycerophosphate	1.41×10^{-2}	-0.09
succinate	1.37 "	+0.009
fumarate	1.44 "	+0.003
malate	1.98 "	-0.02
F6P	2.06 "	-0.38
G6P	2.29 "	-0.25
citrate	2.54 "	-0.06
glucose	3.74 "	-0.08

Table 4.6

The relationship between the responses for the internal standards and the test compounds. Effect of varying the internal standards

The internal standards, hexadecane and docosane, were added in three amounts, 0.016, 0.08 and 0.16 μmole, to groups of silylation mixtures containing TMS-derivatives at the levels shown in the table. The ratio $\frac{\text{area of standard}}{\text{area of TMS-derivative}}$ was calculated for each amount of the standard at a given concentration of the TMS-derivatives. These values were then normalised by making the value for 0.08 μmole of standard equal to 5.00. GLC analysis was carried out on a 3% OV-1 on Gas Chrom Q (100-120 mesh), 5' x ¼" column using the programme mentioned in Table 4.3.

	HEXADECANE									DOCOSANE								
	0.5 μmole			0.2 μmole			0.04 μmole			0.5 μmole			0.2 μmole			0.04 μmole		
Succinate	1.00	5.00	9.8	0.90	5.00	9.6	0.91	5.00	10.3	0.81	5.00	9.9	0.78	5.00	9.9	0.85	5.00	11.2
Fumarate	0.92	5.00	10.2	0.95	5.00	10.3	0.87	5.00	10.3	0.80	5.00	10.4	0.83	5.00	10.4	0.81	5.00	11.2
Malate	0.97	5.00	10.4	0.96	5.00	9.8	1.01	5.00	11.0	0.85	5.00	10.5	0.84	5.00	10.1	0.96	5.00	12.3
α-Glycerophosphate	0.93	5.00	10.3	0.97	5.00	10.6	1.02	5.00	11.6	0.83	5.00	10.3	0.85	5.00	11.0	0.81	5.00	11.5
Citrate	0.98	5.00	10.1	1.00	5.00	10.2	0.99	5.00	11.0	0.85	5.00	10.2	0.92	5.00	10.4	0.93	5.00	11.6
Glucose (α + β)	0.97	5.00	9.5	1.08	5.00	9.9	1.00	5.00	10.8	0.85	5.00	9.6	0.94	5.00	10.1	0.93	5.00	11.5
Fructose-6-Phosphate	0.91	5.00	9.1	1.18	5.00	10.5	1.02	5.00	13.2	0.79	5.00	9.2	1.03	5.00	10.8	0.92	5.00	13.0
Glucose-6-Phosphate	0.96	5.00	9.9	1.03	5.00	10.9	1.00	4.45*	11.2	0.84	5.00	9.9	0.91	5.00	11.1	0.99	5.00	12.7

* only one result available - normalised on 1.00

Table 4.7

The relationship between peak height and peak area during temperature programmed analysis of some TMS-compounds on 3% OV-1 on Gas Chrom Q

The data from chromatograms obtained for samples in Table 4.3 and 4.5 was used to obtain these results. The values presented below are the ratio of the peak area of each compound (calculated by triangulation as sq. cms of chart) to the vertical height of the peak above the baseline (in cms). The ratio was studied at three amounts of each compound in the reaction mixture, 0.5, 0.2 and 0.04 μmole. Values were also obtained for hexadecane and docosane at the amounts, 0.16, 0.08 and 0.016 μmole. Each result was an arithmetical mean ± the standard error of the mean for 8 values (unless otherwise stated).

Gas chromatography was carried out using 3% OV-1 on Gas Chrom Q (100-120 mesh) 5' x ¼" column. The temperature programme was: Isothermal 100°C for 5 minutes, then linear temperature programming at 4°C per minute, 100-250°C. The chart speed of the recorder was 3 cm per minute.

Compound	0.5 μmole	0.2 μmole	0.04 μmole
Succinate	0.76 ± 0.014	0.74 ± 0.018	0.75 ± 0.014 (9)
Fumarate	0.745 ± 0.017	0.76 ± 0.018	0.78 ± 0.011 (9)
Malate	0.72 ± 0.007	0.72 ± 0.006	0.80 ± 0.014 (9)
α-Glycerophosphate	0.71 ± 0.007	0.70 ± 0.004	0.75 ± 0.008 (9)
Citrate	0.705 ± 0.004	0.70 ± 0.006 (7)	0.695 ± 0.005 (9)
Glucose (α+β form)	0.71 ± 0.006	0.695 ± 0.004	0.69 ± 0.004 (9)
Fructose-6-Phosphate	0.78 ± 0.009	0.76 ± 0.013	0.78 ± 0.008 (9)
Glucose-6-Phosphate	0.72 ± 0.004	0.71 ± 0.007	0.76 ± 0.006 (9)
Hexadecane	0.71 ± 0.006 (9)	0.72 ± 0.006	0.74 ± 0.015
Docosane	0.70 ± 0.008 (9)	0.71 ± 0.010 (7)	0.74 ± 0.012

CHAPTER 5

THE DEVELOPMENT OF PROCEDURES FOR THE SILYLATION OF COMPOUNDS IN TISSUE

The previous two chapters have considered the problem of preparing volatile derivatives suitable for gas chromatography, and then separating these derivatives and estimating them. However, there is a big difference between forming derivatives in a mixture of known pure compounds and forming derivatives of compounds in tissue, or in extracts derived from tissue.

In this chapter silylation on freeze dried tissue, on tissue extracts, and on tissue extracts further purified by ion-exchange chromatography has been studied.

A. Direct silylation of tissue

The levels of the intermediates in the tissues isolated were very low when compared with those of the polysaccharides, free sugars, inorganic phosphate and proteins, all of which would be also silylated. Some samples of liver frozen in liquid nitrogen (Wollenberger et al, 1960) were freeze dried, crushed into a powder and further dried in a vacuum desiccator over phosphorus pentoxide. Attempts were made to silylate the samples in 100 µl BSTFA:pyridine:TMCS (2:1:1 by vol). 10 mg of wet tissue (yielding about 2 mg of dry tissue) was the largest quantity which could be silylated satisfactorily and not give a fine suspension which would clog the syringe needle used for injection onto the GLC column. Figure 5.1 shows the chromatogram obtained when 5 µl of the 10 mg sample was injected at attenuation setting x 500. The two major peaks are TMS-α and TMS-β D-glucose. Peaks can be tentatively

(contd. on p.139)

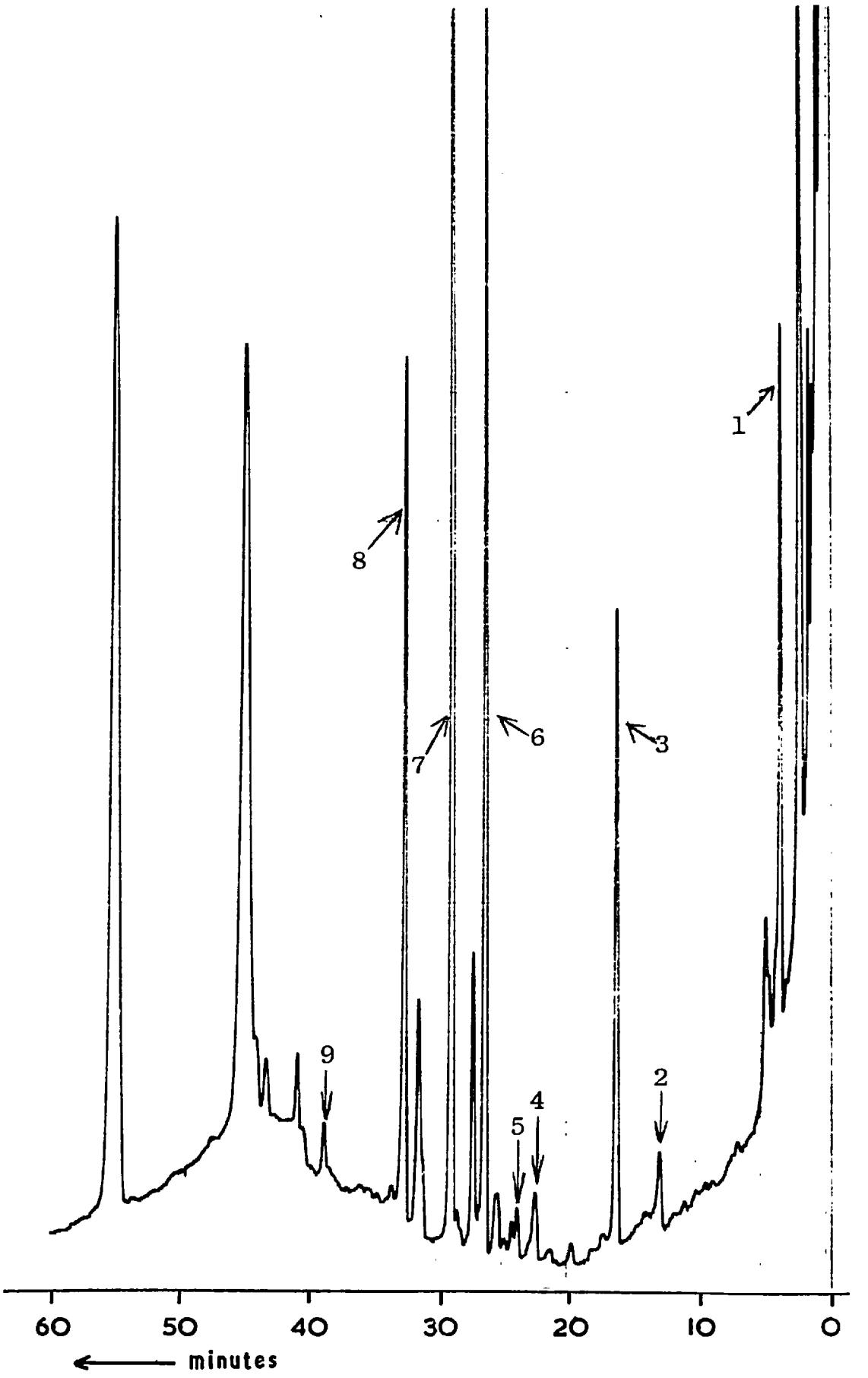

1
2
3
4
5
6
7
8
9
60
50
40
30
20
10
0
minutes

Figure 5.1 Direct silylation of 10 mg of liver after freeze drying

10 mg of liver which had been freeze clamped in liquid nitrogen (Wollenberger et al, 1960) was freeze dried. It was powdered and further dried over phosphorous pentoxide under vacuum. It was then treated with 100 µl of silylation reagent (BSTFA: pyridine:TMCS, 2:1:1 by vol).

5 µl of this was injected onto a 5' x ¼", 3% OV-1 on Gas Chrom Q (100-120 mesh) column using the temperature programme: 100°C for 5 minutes, then linear temperature programming at 4°C per minute, 100-250°C.

Tentative peak identifications based on retention times are as follows:

1. phosphate 2. malate 3. hexadecane 4. α-glycerophosphate
5. citrate 6. α-D-glucose 7. β-D-glucose 8. docosane
9. glucose-6-phosphate

identified for malate, α-glycerophosphate, citrate and glucose-6-phosphate. However, this is not a very satisfactory method, the limit of 10 mg makes it hardly suitable for mass analysis, and not at all suitable for radioactivity analyses since there would be insufficient for measurements.

B. Silylation of tissue extracts

If simply an extract of tissue were to be silylated the extractant would have to be readily removed. Perchloric acid (7.2% w/v) whilst being an effective protein precipitant was difficult to remove. All the last traces had to be removed before silylation, precipitation as its potassium salt at 0°C was not sufficient. In addition it left large amounts of polysaccharide in solution, a greater problem with regards to silylation than having protein present.

The best extractants were the volatile ones, and of these aqueous ethanol was chosen as it extracted at neutral pH. The optimum concentration of ethanol to give the highest extraction and at the same time greatest precipitation of protein and polysaccharide was 60%. The compound most sensitive to high concentrations of ethanol, FDP, was added to portions of tissue at -20°C which were then extracted with various aqueous ethanols. The recoveries of FDP, as [U-^{14}C]-FDP, are shown in Figure 5.2. 60% aqueous ethanol gave a much clearer supernatant after centrifugation than 40% or 50% aqueous ethanol. This was more apparent when the solvent was removed, the least residue being with 60% aqueous ethanol. Extraction figures as the percentage recoveries of the ^{14}C-labelled compounds were determined for G1P, G6P, F6P and FDP using 50%, 60% and 80% aqueous ethanol and also 7.2% perchloric acid as a reference (see Table 5.1).

(contd. on p.143)

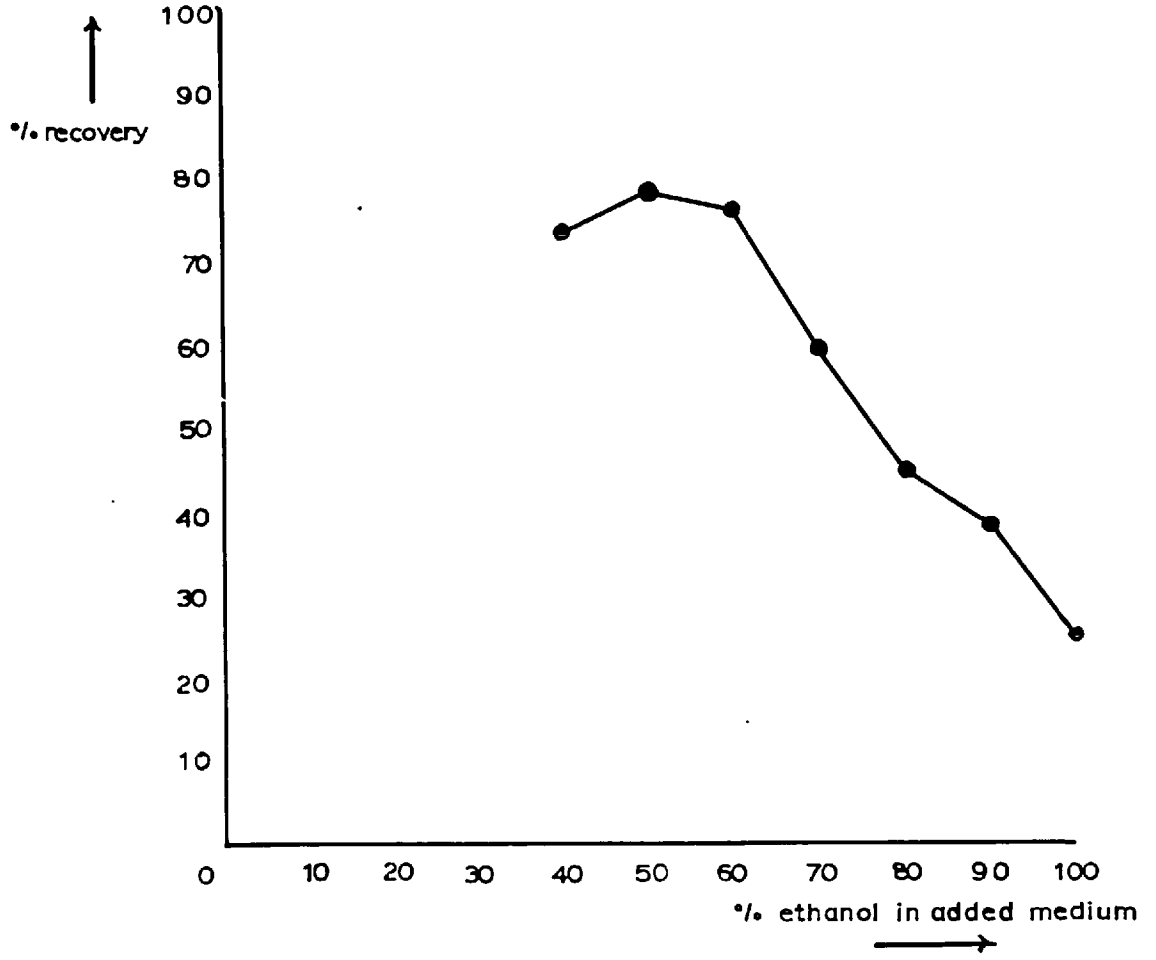

% recovery
100
90
80
70
60
50
40
30
20
10
0
10
20
30
40
50
60
70
80
90
100
% ethanol in added medium

Figure 5.2 The recoveries of [U-^{14}C]-labelled FDP from plasmodia by extraction with aqueous ethanols

[U-^{14}C]-labelled FDP (1 μc, 1 μmole) was added to each of seven 2.5 g portions of frozen powdered plasmodia, and mixed. Extraction was carried out with aqueous ethanol in concentrations of 40%-100% (v/v) on separate samples as for Table 5.1.

Table 5.1

The recovery of added ^{14}C-labelled sugar phosphates from rat heart and P.polycephalum plasmodia by extraction with aqueous ethanol

The ^{14}C-labelled sugar phosphates (1 μmole, 1 μCi) were added to 2-3 g portions of powdered frozen plasmodia or rat heart, and the powder mixed. The powder was extracted twice with 4 volumes of the solvents as described in Methods. Aliquots of the extracts were taken for determination of radioactivity.

$$\% \text{ recovery} = \frac{\text{dpm recovered}}{\text{dpm added}} \times 100\%$$

% recovery in each solvent

Sugar Phosphate	50% ethanol	60% ethanol	60% ethanol	80% ethanol	7.2% PCA
	plasmodia	heart	plasmodia	plasmodia	plasmodia
[U-^{14}C]-G1P	98, 99	97, 100	100, 95	93, 91	94
[U-^{14}C]-G6P	89, 91	88, 92	85, 89, 88	82, 80	89
[U-^{14}C]-F6P	n.m.	n.m.	93, 94	n.m.	n.m.
[U-^{14}C]-FDP	78, 82	87, 81	83, 80	37, 34	80

n.m. = not measured

PCA = perchloric acid

Frozen pulverised liver (1 g) was extracted at -5°C with 2 x 10 ml 60% aqueous ethanol and extracts corresponding to 10 mg and 100 mg of liver evaporated to dryness. These were silylated with 100 µl of BSTFA:pyridine:TMCS (2:1:1 by vol). The extract of 100 mg of liver was about the limits of material that could be used without blocking the syringe. However, with rat heart tissue larger amounts could be used because of the lower proportion of polysaccharides. Figure 5.3 shows the chromatogram obtained by injecting 5 µl of the extract of 10 mg liver. Again TMS-α- and TMS-β-D-glucose were the major peaks; extra peaks could be seen and the baseline movement was less pronounced. For the sample corresponding to an extract of 100 mg of liver the chromatogram in Figure 5.4 was obtained. This chromatogram of 10 times the amount of liver was at 10 times less sensitivity than those in Figures 5.1 and 5.3. The individual compounds can be seen more clearly, the baseline being much more stable. It is conceivable that mass measurements could be made with this amount of liver.

However, for measurements of radioactivity in the individual compounds at least a gramme of tissue would be needed so that accurate measurements could be made. This would mean that 1000 µl of the silylation reagent would be needed which is wasteful and expensive. Also large volumes would have to be injected onto the column to get sufficient radioactivity in each compound. Thus a greater amount of purification of the tissue extract was needed.

C. Purification of the aqueous ethanol extract necessary for silylation

As the radioactive studies involving incubation of tissue were to be made with ^{14}C-labelled glucose, and as liver as extracted

(contd. on p.148)

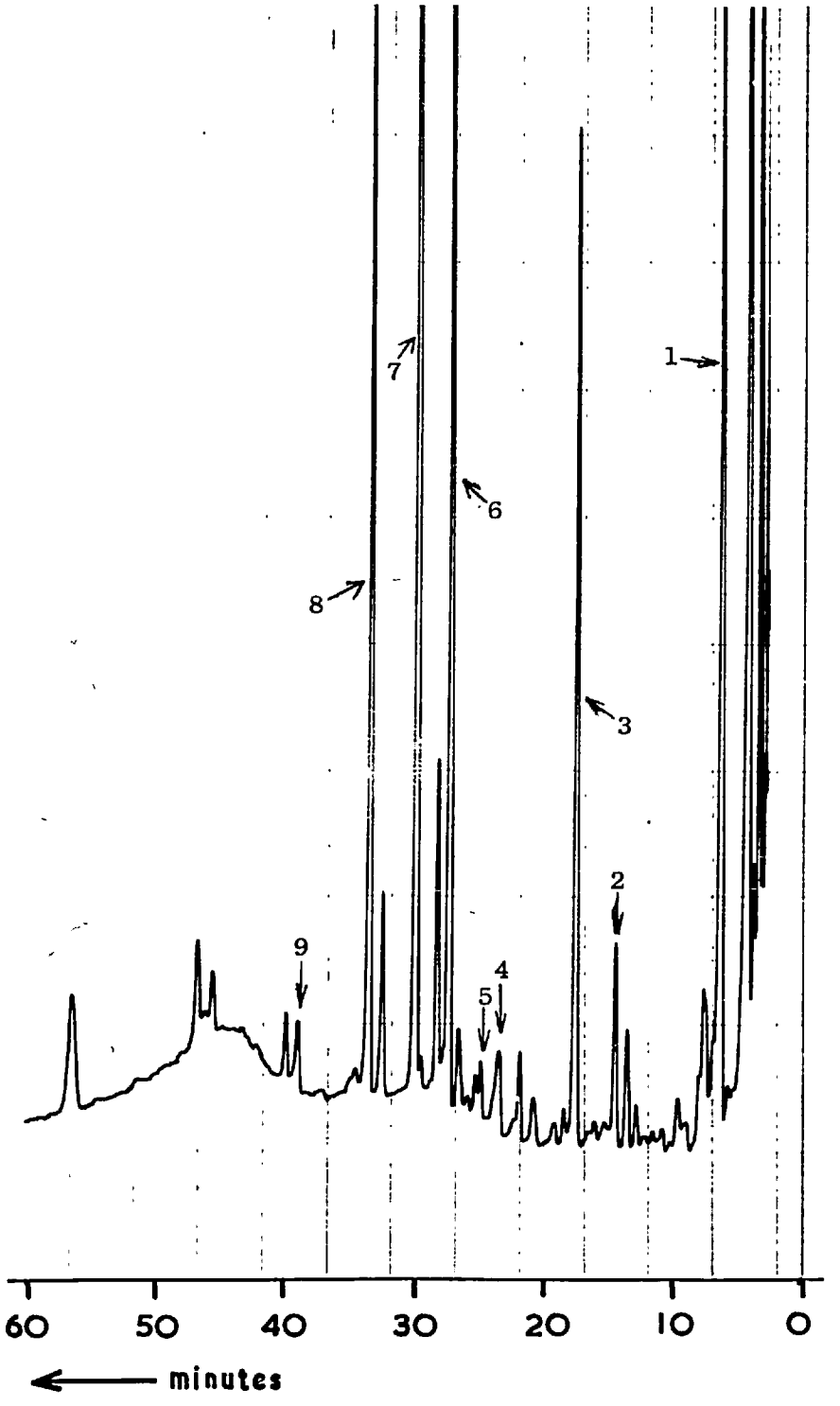

1
7
6
8
3
2
9
4
5
60
50
40
30
20
10
0
minutes

Figure 5.3 Silylation of 10 mg of liver after ethanol extraction

10 mg of liver which had been freeze clamped in liquid nitrogen (Wollenberger et al, 1960) was extracted with 2 x 1 ml 60% aqueous ethanol. The extract was evaporated to dryness under vacuum and over phosphorous pentoxide. It was treated with 100 µl silylation reagent (BSTFA:pyridine:TMCS, 2:1:1 by vol).

5 µl of this was injected onto a 5' x ¼", 3% OV-1 on Gas Chrom Q (100-120 mesh) column, using the temperature programme: 100°C for 5 minutes, then linear temperature programming at 4°C per minute, 100-250°C.

Tentative peak identifications based on retention times are as follows:

1. phosphate 2. malate 3. hexadecane
4. α-glycerophosphate 5. citrate 6. α-D-glucose
7. β-D-glucose 8. docosane 9. glucose-6-phosphate

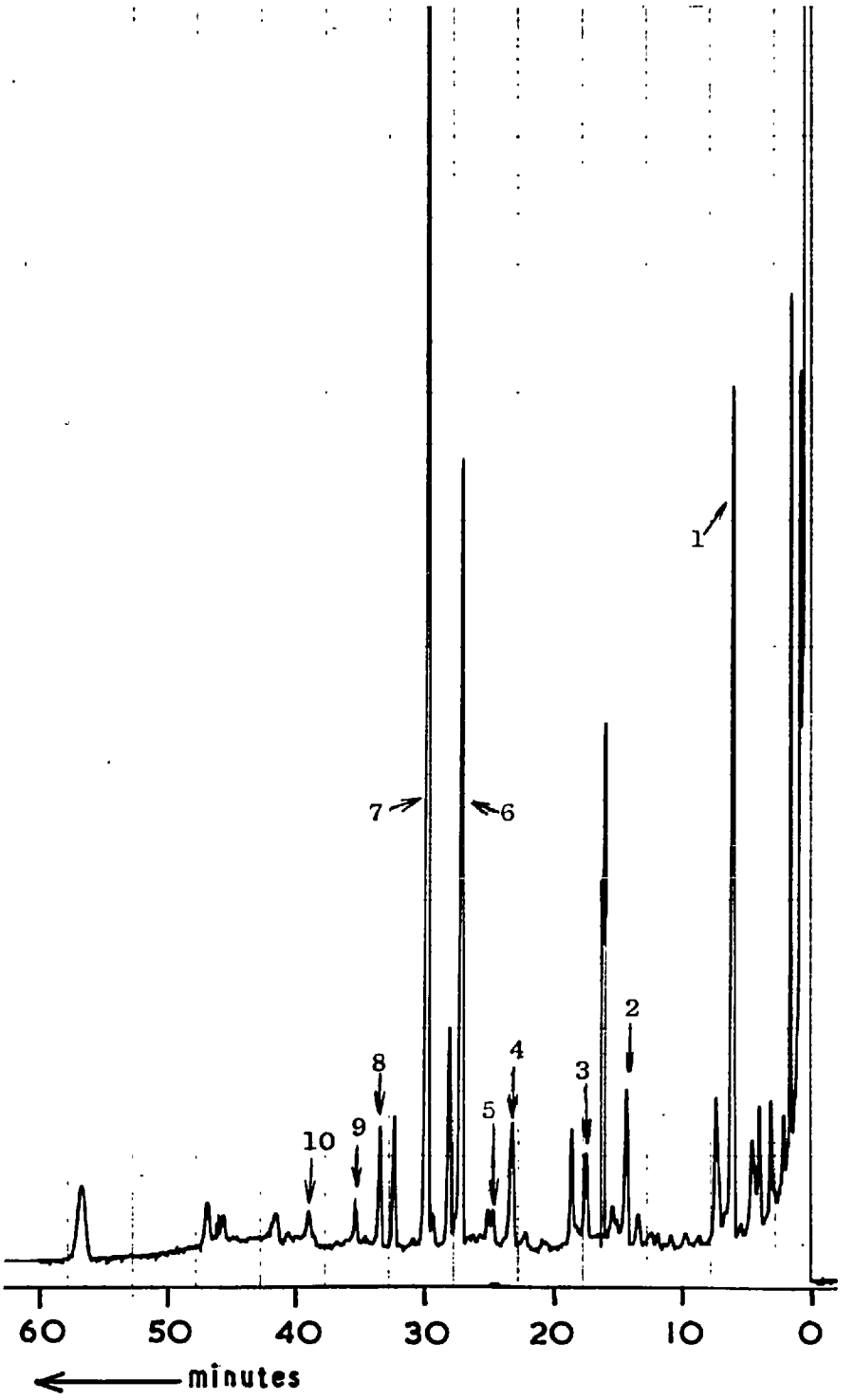

1
2
3
4
5
6
7
8
9
10
60
50
40
30
20
10
0
minutes

Figure 5.4 Silylation of 100 mg of liver after ethanol extraction

100 mg of liver was treated as described in Figure 5.3. 5 µl of the silylation reaction mixture was analysed at ten times less sensitivity of detection (to counteract the ten times increased mass).

Tentative peak identifications based on retention times are as follows:

1. phosphate 2. malate 3. hexadecane 4. α-glycerophosphate
5. citrate 6. α-D-glucose 7. β-D-glucose 8. docosane
9. fructose-6-phosphate 10. glucose-6-phosphate

contained very large amounts of glucose (see Figures 5.1, 5.3 and 5.4) compared with the intermediates to be studied, an important step in a purification procedure would be the removal of free glucose. Similarly for the incubations of plasmodia since they could not be centrifuged down quickly enough, aliquots of medium containing the suspended plasmodia were sampled and frozen. The frozen lumps were treated as if they were all tissue and this further increased the ratio of glucose to the intermediates. In addition, large amounts of inorganic phosphate were present and this would be an interference when silylation was carried out.

1) Removal of inorganic phosphate from tissue extracts

Since phosphate was a major contaminant all water used in the purification of the extract was deionised prior to use. Phosphate was removed immediately after tissue extraction since it was not convenient to do so later and because the method eventually used effectively clarified the extract. This was most noticeable with liver extracts.

Precipitation of phosphate as barium phosphate in 50% aqueous ethanol was not satisfactory since some co-precipitation of the sugar phosphates, particularly FDP, occurred. However, precipitation as magnesium ammonium phosphate was successful since no losses could be detected for the sugar phosphates, G6P, G1P, F6P and FDP (1 μmole present). Since the tissue extracts would be unlikely to contain greater amounts than 1 μmole no losses should be expected. In fact no losses of radioactivity were detected when inorganic phosphate was precipitate from extracts of plasmodia incubated with ^{14}C-glucose (incubated until the external concentration of glucose fell to zero, i.e. all soluble radioactivity present as intermediates, not as free glucose).

However it was important that the pH should not rise above 10 as magnesium hydroxide would start to be precipitated. This caused a sudden fall off in the recovery. This is shown clearly for F6P in Figure 5.5.

2) Removal of polysaccharides and free sugars from the tissue extract by ion-exchange chromatography

The contaminants remaining in the extract at this stage included neutral sugars, polysaccharides and amino acids. In all metabolic experiments the most important contaminant was glucose. Since it was in vast excess to the sugar phosphates it was essential to remove it completely.

Since all the intermediates to be assayed were organic acids, separation from the neutral sugars by selective absorption onto an anion exchange resin was investigated. With the resin in the borate form elution with 2M formic acid, whilst giving a separation between glucose and the sugar phosphates, resulted in bad tailing of the glucose (see Figure 5.6A). With the resin in the formate form and if 0.2M formic acid was used instead of 2M formic acid as the initial eluting agent, glucose was effectively removed with only slight elution of G1P (see Figure 5.6B). It was noticed that eluting with small increments at a time rather than loading the reservoir with all the eluting agent, reduced tailing of the peaks.

When the resin was in the borate form, water did not elute glucose at all. However, with the resin in the formate form glucose was eluted with water immediately, and the sugar phosphates completely retained. The sugar phosphates were not eluted by 0.2M formic acid, but were readily eluted by 2.0M formic acid or 0.5M sodium formate.

(contd. on p.154)

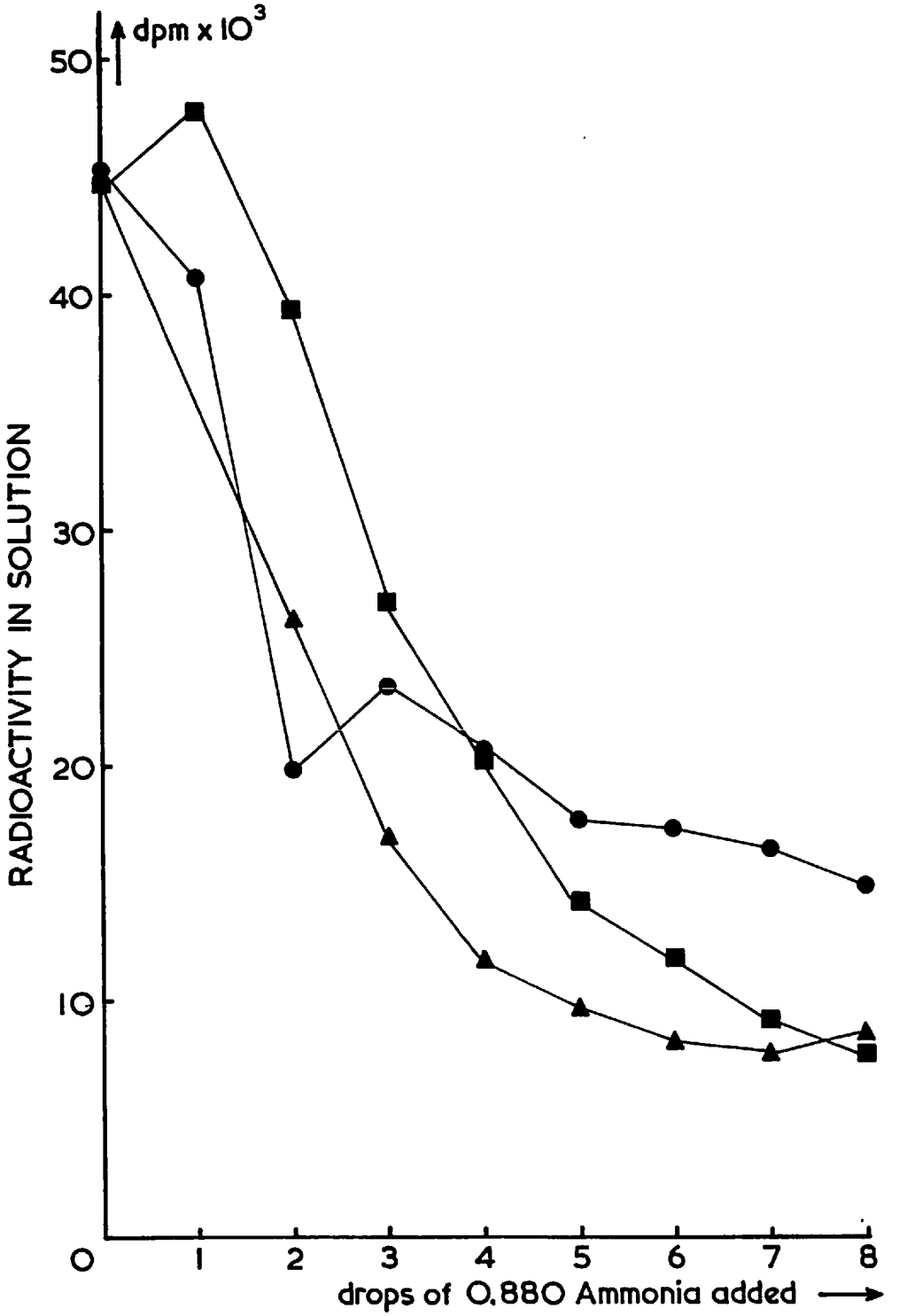

dpm x 10³
50
40
30
20
10
0
RADIOACTIVITY IN SOLUTION
1
2
3
4
5
6
7
8
drops of 0.880 Ammonia added

Figure 5.5 The effect of excess ammonia during the removal of phosphate on the solubility of F6P

[U-^{14}C]-F6P (1 μmole, 2 μCi) was dissolved in 5 ml water with 1 μmole each of succinate, fumarate, malate, α-glycerophosphate, citrate, glucose and G6P. Three such solutions were made up and 1 ml magnesium acetate (containing 30 mg) added to each. To the first 0.2 ml distilled water was added, to the second 0.2 ml 15 mM potassium phosphate buffer, and to the third 0.2 ml 200 mM potassium phosphate buffer. Each was carefully titrated to pH 10 with ammonium hydroxide and the volumes adjusted so that they were equal. The precipitate of magnesium ammonium phosphate was centrifuged (2000 g for 10 minutes) and an aliquot of the supernatant removed for radioactivity determination. One drop of 0.880 ammonium hydroxide was added, the samples shaken and centrifuged. Another aliquot of the supernatant was removed. Seven further drops of 0.880 ammonium hydroxide were added dropwise, the soluble radioactivity being determined at each stage.

Identification:

- ● F6P + 0.2 ml distilled water
- ▲ F6P + 0.2 ml 15 mM potassium phosphate buffer
- ■ F6P + 0.2 ml 200 mM potassium phosphate buffer

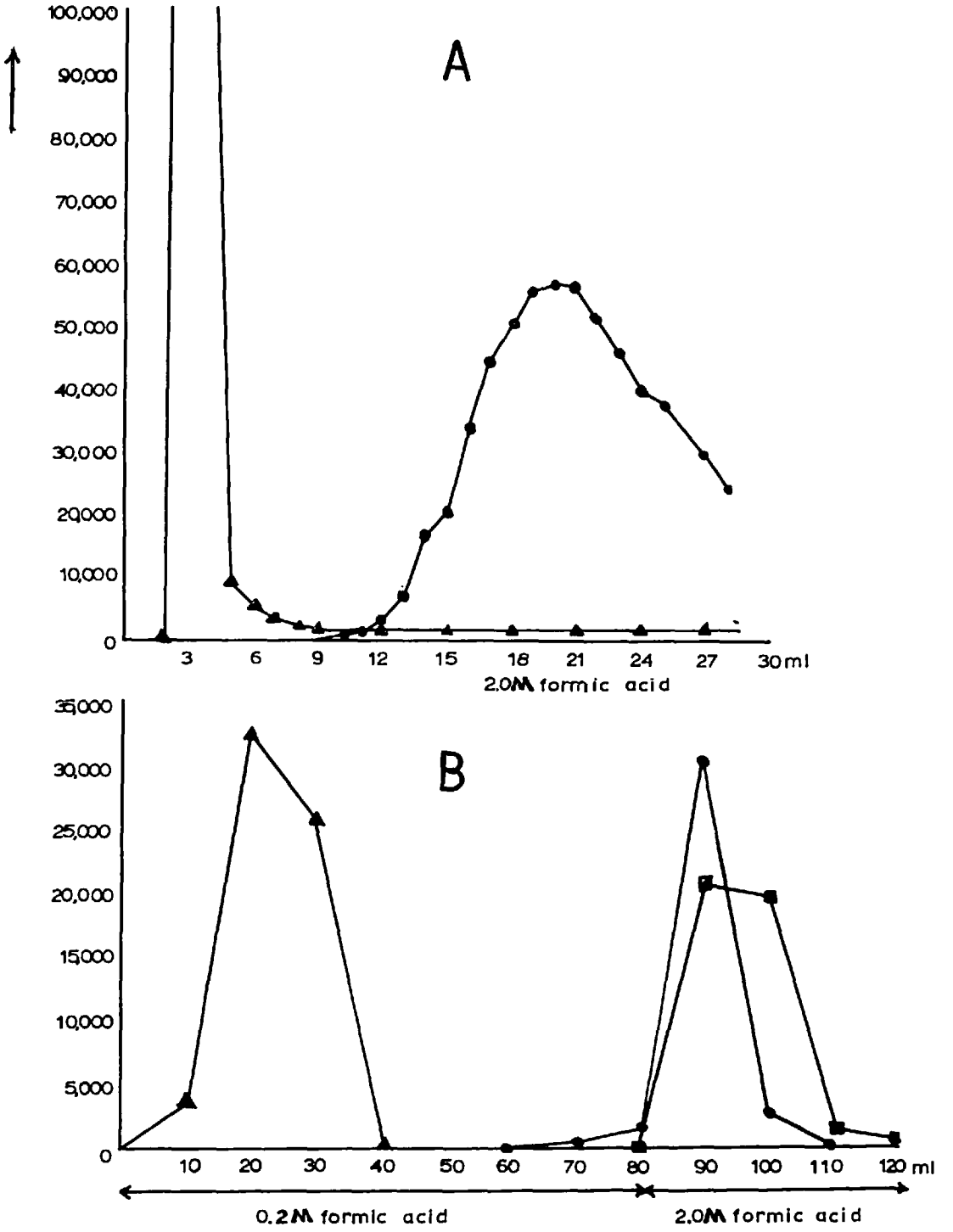

A
100,000
90,000
80,000
70,000
60,000
50,000
40,000
30,000
20,000
10,000
0
3
6
9
12
15
18
21
24
27
30ml
2.0M formic acid
B
35,000
30,000
25,000
20,000
15,000
10,000
5,000
0
10
20
30
40
50
60
70
80
90
100
110
120 ml
0.2M formic acid
2.0M formic acid

Figure 5.6 Separations of [U-^{14}C]-glucose and [U-^{14}C]-sugar phosphates in AG 1 resin columns

AG 1 resin (chloride form, 100-200 mesh) was packed into 12 x 0.5 cm columns.

In Figure A triethylammonium borate (0.5M pH 8.4) was passed through the resin column to convert it to the borate form. It was washed with distilled water to remove the borate. In separate runs [U-^{14}C]-glucose and [U-^{14}C]-glucose-6-phosphate were carefully loaded onto the top of the column. 2M formic acid was added to the reservoir and 1 ml fractions eluted from the column were collected, and the radioactivity in them determined.

▲ [U-^{14}C]-glucose

● [U-^{14}C]-glucose;6-phosphate

In Figure B sodium formate (2M) and formic acid were passed successively through the resin column to convert it to the formate form. Distilled water was passed through the column until the eluate was neutral. In separate runs [U-^{14}C]-glucose, [U-^{14}C]-glucose-1-phosphate and [U-^{14}C]-glucose-6-phosphate were carefully loaded onto the top of the column. 0.2M formic acid (10 ml) was added to the reservoir. Once the level of fluid reached the top of the resin a further 10 ml was added. Six further amounts of 10 ml were added to the reservoir. Eluate fractions were the corresponding 10 ml added. Then 4 x 10 ml 2M formic acid was passed through the column. Aliquots of each fraction were used for the determination of radioactivity.

▲ [U-^{14}C]-glucose

● [U-^{14}C]-glucose-1-phosphate

■ [U-^{14}C]-glucose-6-phosphate

Considerable amounts of radioactivity were present in 2M formic acid and 0.5M sodium formate eluates from resin columns in the formate form from which glucose had been previously eluted by water. To check whether this was due to impurities in the glucose, or glucose interacting with active sites in the resin, two identical AG 1 columns in the formate form were prepared. [U-^{14}C]-glucose was passed through the first column and the first 10 ml aqueous eluate collected, divided into two, and 5 ml transferred to the second column. Both columns were then eluted with water, 0.2M formic acid and 0.5M sodium formate as in Table 5.2. Since an impurity was found to be present which would contaminate the sodium formate elution fraction containing the sugar phosphates, all ^{14}C-glucose used in experiments from this point was purified by passage through a small AG 1 formate resin column.

3) The recovery of ^{14}C-labelled intermediates from the AG 1 resin column (formate form)

0.5M sodium formate was generally preferred as the eluting agent despite the ready removal of formic acid under reduced pressure. This was because FDP was not eluted at all by any concentration of formic acid. 0.5M sodium formate was the lowest concentration needed to elute it in a reasonable volume (40 ml). As shown in Table 5.3 the other sugar phosphates and citrate, fumarate, malate and succinate were eluted with this reagent. The recoveries, except for FDP, were near to 100% since the ^{14}C-labelled materials were not purified before use.

(contd. on p.157)

Table 5.2

The contamination of commercial [U-^{14}C]-glucose

Two AG 1 resin columns (12 x 0.5 cm) in the formate form were used. [U-^{14}C]-glucose (8 μmole, 8 μC$_i$) in solution was passed through column A, washing with 10 ml distilled water. The first 10 ml collected was divided into two, and one portion applied to column B. Both columns were further washed as indicated in the table. 10 ml amounts of the eluting agent were added at a time to the column reservoir.

Elution medium	Column A dpm/ml	Column B (purified glucose) dpm/ml
4 x 10 ml water	223,116	239,237
next 10 ml	21	7
4 x 10 ml 0.2M formic acid	3,313	14
next 10 ml	- - - -	3
4 x 10 ml 0.5M sodium formate	1,176	6
next 10 ml	- - - -	2

Table 5.3

Percentage recoveries of various intermediates from AG 1 formate form resin

Each of the ^{14}C-labelled compounds (1 μmole, 1 μC_i) were added to 2 g of frozen powdered plasmodia and the powder extracted twice with 60% aqueous ethanol (4 volumes). Each extract was applied to an AG 1 column (12 x 0.5 cm) in the formate form. The column was washed with 10 x 10 ml distilled water and then the compounds eluted with 4 x 10 ml 0.5M sodium formate.

Compound	% recovery
G6P	98.7+
G1P	99.5+
FDP	93.3+
F6P	96.8*
Citrate	96.0*
Fumarate	97.2*
Malate	97.7*
Succinate	100.0*

\+ mean of three values

* mean of two values

4) The concentration and purification of the sodium formate eluate

The sodium ions were removed by passing the eluate (40 ml) through an AG 50 (H^+ form) column (30 x 2.5 cm). The column was washed with a further 40 ml deionised distilled water. This eluted greater than 90% of all the compounds studied, the remaining 10% only being eluted by vastly increasing the elution volume. The resulting formic acid solution was evaporated to dryness under vacuum at 30°C. Because of the risk of loss of volatile acids the flask was removed from the rotary evaporator as soon as dryness had been achieved.

In the experiments where 2.0M formic acid was used as an eluant, an orange pigment was eluted from myxomycete plasmodial extracts which was removed before evaporation of the eluate to dryness. This pigment was particularly difficult to remove because of its ionic character. In acid conditions, however, it could be extracted into diethyl ether, ethyl acetate, sec-butanol or tert-amyl alcohol. Diethyl ether and ethyl acetate were used with these formic acid eluates. A check with citrate, fumarate, malate and succinate showed that they were extracted to differing extents by diethyl ether or ethyl acetate (see Table 5.4), citrate and malate hardly at all, fumarate and succinate considerably.

The 2M formic acid eluates were also passed through a small AG 50 (H^+ form) column prior to evaporation to dryness. The dried residues were taken up in 33% aqueous ethanol. At one stage 80% ethanol was used, this preventing some white solid going into solution, but it was found that losses occurred with FDP. Water was not used because the small amount of fluid present formed droplets which were not quantitatively removable from the flask. Triethylamine was added to the extract to prevent the loss of volatile acids such as lactic acid during evaporation to dryness in the specimen tube.

(contd. on p. 159)

Table 5.4

The removal of some Krebs cycle intermediates from 2M formic acid by diethyl ether and ethyl acetate

^{14}C-labelled compounds (2 μmole, 1 μc) were added to 40 ml 2M formic acid in a separating funnel (100 ml). Diethyl ether or ethyl acetate (40 ml) was added and the funnel shaken. Aliquots of the upper and low phases were taken (to give the partition ratio). The solvent phase was removed and the aqueous phase extracted twice more. The final aqueous phase, and the three solvent phases after combining and concentrating were sampled (to give ratio between aqueous phase and combined solvent washings).

Compound	Partition ratio		Ratio between aqueous phase and all solvent washings	
	Diethyl Ether	Ethyl Acetate	Diethyl Ether	Ethyl Acetate
Citrate	61	31	20	10
Fumarate	2.0	3.3	0.44	0.82
Malate	86	14	29	4.3
Succinate	—	0.97	—	0.14

The recoveries of intermediates from tissue as far as this stage, i.e. extraction with 60% aqueous ethanol, the precipitation of phosphate, the purification using ion-exchange AG 1 and AG 50 resins and the evaporation to dryness in small specimen tubes prior to silylation, are summarised in Table 5.5.

Table 5.5

The percentage recovery of some ^{14}C labelled tissue intermediates from rat heart and P.polycephalum plasmodia after solvent extraction from the tissue and purification of this extract for gas chromatography

Each intermediate (1 μc; 1 μmole) was added to frozen powdered plasmodia or heart (about 2.5 g) and extracted twice with 10 ml cold 60% aqueous ethanol. The combined extracts were neutralised with 0.880 ammonium hydroxide and evaporated to dryness. The inorganic phosphate present was precipitated as magnesium ammonium phosphate. The extract was absorbed onto an AG 1 formate column which was washed with 9 x 10 ml distilled water. The intermediates were eluted with 4 x 10 ml sodium formate (0.5M). The combined eluate was passed through an AG 50 (H^+ form) column, washing the column with a further 40 ml water. The eluate and washings were combined and evaporated to dryness. The dry solid was eluted with 3 x 1 ml 33% aqueous ethanol and transferred to a specimen tube (75 x 12 mm). The liquid was removed under vacuum over phosphorus pentoxide. The ratio of the radioactivity present in the dry sample to that added originally was the recovery. Each sample when trimethylsilylated and subjected to GLC analysis gave rise to a single peak except for FDP and G1P. G1P gave two peaks which corresponded to α- and β-glucose. FDP was not chromatographed successfully.

Compound	Plasmodia	Rat Heart
G1P	82, 81	85
G6P	82, 80	85
F6P	84, 81	—
FDP	66, 79.5, 66	75
citrate	95, 95	—
fumarate	88, 99	—
malate	88, 97	—
succinate	94, 94	—

CHAPTER 6

APPLICATION OF GLC METHODS TO THE DETERMINATION OF COMPOUNDS FROM TISSUE

In Chapter 4 the determination of the intermediates in standard solutions by GLC was developed, and in Chapter 5 methods for extracting and purifying these intermediates from tissue were examined. These examinations were carried out using ^{14}C-labelled compounds and covered extraction of tissue, and purification as far as the dried solids awaiting silylation.

In this chapter the recovery of ^{14}C-labelled compounds through the entire method, including silylation and elution from the GLC column into the scintillation cell, has been studied. In addition the mass recoveries of intermediates added to tissue extracts have been determined. Interference by contaminating compounds has also been examined. Finally the method used for measurement of mass and radioactivity of intermediates in tissue is laid out in detail.

1) The recovery of ^{14}C-labelled intermediates from tissue

The recoveries of ^{14}C-labelled intermediates as far as the dried solids ready for silylation were in the range 80-95% except for FDP (60-80%) (see Table 5.5). Recoveries of so-called 'pure' ^{14}C-labelled citric acid, F6P and G6P from the GLC column were previously found to be about 90% when using the scintillation cell (see Table 3.10). If no interference occurred in the samples extracted from tissue then final recoveries of 70-90% would be anticipated. This in fact was what was found when 1 umole (1 μC_i) of each intermediate was added to

2.5 g of powdered tissue, and then taken through the entire procedure (see Table 6.1). Only radioactivity associated with the mass peak of the compound under test was used for the calculation of recovery. This radioactivity was corrected for the splitting of the sample and for the efficiency of counting, and after correction for the amount injected onto the GLC column, was compared with that originally added to the powdered tissue.

The accuracy of the specific radioactivity estimation relied on all the radioactivity associated with a given mass being eluted from the GLC column. The recoveries from the GLC column in Tables 3.11 and 3.12 were about 90%. There is every likelihood however that for the amount of sample being injected (50 nmoles) that the recoveries were closer to 100%. The radio purity of the ^{14}C-labelled compounds was in doubt. It was noticed that for [U-^{14}C]-F6P and [U-^{14}C]-G6P that 4-5% of the radioactivity present was not retained by the AG 1 resin column (formate form) despite these compounds being chromatographically 'pure' by paper chromatographic methods. Also recoveries of samples purified from tissue extracts just through the final radioactive GLC stage, i.e. comparison of the amount of radioactivity injected onto the column and the radioactivity eluted off it, were in the range 95-100%. This was also true of samples arising from incubations of tissue with ^{14}C-labelled glucose. Similar anomalies have been reported for some ^{14}C-labelled amino acids (Chou et al, 1970) where bidimensional paper chromatography both by the isotope manufacturer and the authors indicated a purity greater than 98%, whereas ion-exchange elution analysis showed the presence of impurities ranging from 1% to 20% depending on the source of the isotope.

(Contd. on p. 164)

Table 6.1

The percentage recoveries of ^{14}C-labelled compounds, which were added to tissue, after extraction from the tissue, partial purification by ion-exchange analysis, formation of trimethylsilyl derivatives, and separation by gas-liquid chromatography

Each compound (1 μmole, 1 μC_i) was added to frozen powdered tissue (2.5 g) and extracted with 2 x 10 ml 60% aqueous ethanol. The phosphate present was removed as a precipitate of magnesium ammonium phosphate. The extract was loaded onto an AG 1 (formate form) resin column (12 x 0.5 cms) and the column washed with 9 x 10 ml water. The intermediates were eluted with 4 x 10 ml sodium formate (0.5M) and the eluate passed through an AG 50 (H^+ form) resin column (30 x 2.5 cms), washing the column with a further 40 ml water. The combined eluate was evaporated to dryness and transferred to a specimen tube (75 x 12 mm) by 3 x 1 ml 33% aqueous ethanol. It was evaporated to dryness overnight over phosphorus pentoxide under vacuum. After converting the acids present to their triethylammonium salts, the sample was treated with BSTFA:pyridine:TMCS (2:1:1 by vol) at 4°C. Aliquots (5 μl) were injected onto a 5' x ¼" OD glass column containing 3% SE-52 on Chromosorb W (AW-DMCS, 80-100 mesh). A stainless steel splitter (1:1) was fitted onto the end of the column. One half of the column effluent was passed into the scintillation cell where the radioactivity was continuously monitored. The radioactivity associated with the peak due to the compound under test was corrected for splitting and counting efficiency (F = 3.64) and compared with that initially added to the tissue.

Compound	Plasmodia	Rat Heart
G6P	75, 77	79
F6P	74	77
G1P	80, 84	84
citric acid	86	—
fumaric acid	84	—
malic acid	84	—
succinic acid	85	—

2) The recovery of intermediates added to tissue

a. Samples taken through the purification procedures

When making measurements of specific radioactivity it is important that both the mass and radioactivity of the compound being studied be eluted to the same extent. Since the recoveries of radioactivity from tissue found in Table 6.1 were for radioactivity associated with mass peak, and that only one radioactive compound was studied at a time, it would be expected that for these samples mass and radioactivity were eluted to the same extent. It is also unlikely that if any losses occur during the work-up and analysis that the non-radioactive material should behave any differently from the radioactive material.

However, since the amount of each intermediate added in the radioactivity recovery studies (1 μmole) was in the higher physiological range it was of interest to measure recoveries of mass of different amounts of each intermediate and in the presence of differing amounts of tissue. The results of these measurements are shown in Table 6.2. Recoveries of the 500 nmoles were 70% or greater for all intermediates except F6P in the presence of the most tissue (500 mg liver or 175 mg heart). With less tissue (liver 50 mg and heart 17.5 mg) many recoveries were lower than 70%, the heart data being lower than the liver data in most cases. It would seem from this data the amount of tissue has a marked effect on the recovery by blocking 'active sites' possibly on glassware, ion-exchange resins, etc. Indeed the recovery of intermediates (500 nmoles) in the absence of any added tissue was less than 30% measured on two separate occasions. By extrapolating up to the conditions used for measurement of radioactivity recovery, i.e. 2.5 g tissue and 1 μmole of each intermediate, recovery of mass and radioactivity are essentially the same.

(contd. on p.166)

Table 6.2

The percentage recoveries of the mass of intermediates added in two different amounts (500 nmoles and 200 nmoles) to differing amounts of tissue extracts

A solution containing 500 nmole per ml of succinate, fumarate, malate, ∝-glycerophosphate, citrate, fructose-6-phosphate and glucose-6-phosphate was added (1 ml and 0.4 ml) to liver extracts (500 and 50 mg wet weight) and heart extracts (175 and 17.5 mg wet weight). Each sample was purified as for the legend to Table 6.1. The resulting extract was treated with triethylamine (3 drops) and re-evaporated to dryness. Silylation reagent (0.1 ml) containing BSTFA:pyridine:TMCS (2:1:1, by vol) was added. The pyridine contained the internal standards n-hexadecane and n-docosane. A standard curve of area response against concentration was determined using the same stock solution of the intermediates and the same silylation reagent. Gas chromatographic analysis was carried out by injecting 5 µl aliquots onto a 3% OV-1 on Gas Chrom Q (100-120 mesh) 5' x ¼" column using the temperature programme: 100°C isothermal for 5 minutes, then linear temperature programming at 4°C per minute, 100-250°C. The amount of each intermediate present in the test solutions was corrected for the amount in the tissue extracts without any additions.

Compound	Tissue	500 nmoles		200 nmoles	
		10 vols	1 vol	10 vols	1 vol
SUCCINATE	Liver	120	131	55	88
	Heart	93	77	92	44
FUMARATE	Liver	87	78	60	28
	Heart	74	39	47	30
MALATE	Liver	70	78	28	56
	Heart	75	45	75	44
∝-GLYCEROPHOSPHATE	Liver	129	109	109	95
	Heart	88	52	98	22
CITRATE	Liver	69	40	53	29
	Heart	78	36	21	12
FRUCTOSE-6-PHOSPHATE	Liver	36	-	50	30
	Heart	50	39	39	-
GLUCOSE-6-PHOSPHATE	Liver	75	43	64	28
	Heart	68	64	71	28

For the radioactive gas chromatographic method to have enough radioactivity in each peak, fairly large samples of tissue, certainly not less than 1 g, would be needed. This would avoid the problems created by the drop in recovery with small amounts of tissue. Indeed to measure the mass in small amounts of tissue an alternative method described in Chapter 5 could be used.

b. Samples not purified before silylation

As suggested in the previous chapter, it might well have been possible to measure the level of carbohydrate intermediates in tissue without the purification procedure. That is to extract the tissue with aqueous ethanol, and then to simply evaporate the extract to dryness in a specimen tube. This dry sample could then be silylated. This was the procedure used to give the chromatograms in Figures 5.3 and 5.4.

Since the impurities normally removed were still present in the aqueous ethanolic tissue extract it was necessary to see whether they interfered with the silylation of the carbohydrate intermediates. To test this aliquots of liver extract of 0.1, 0.4 and 1.0 ml (representing about 5, 20 and 50 mg wet weight of liver tissue) were taken, and aliquots of a standard mixture of carbohydrate intermediates (50, 200 and 500 nmoles) added. Each was dried down in vacuo and then silylated.

The results presented in Figure 6.1 showed quite clearly that for all the compounds that were added tissue did interfere with the response. This could be most ably demonstrated when the slope of the response curve of a compound was plotted against the amount of tissue extract present. Even when 0.1 ml of liver tissue extract (5 mg of

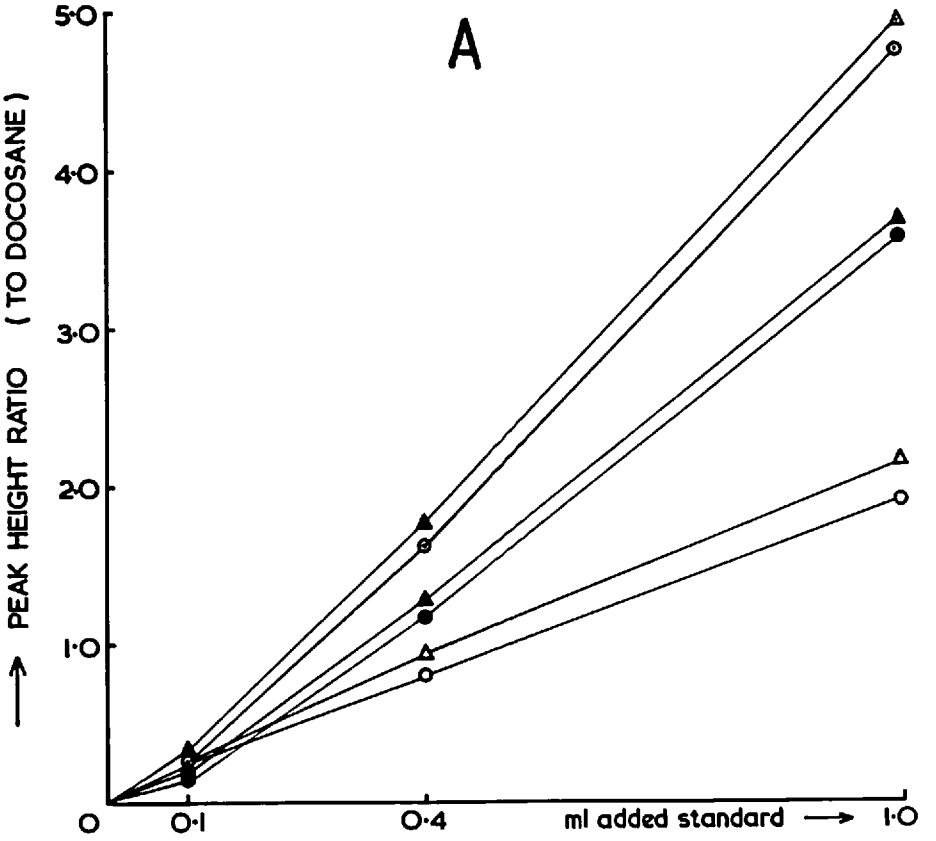
liver) was present there was an interfering effect (see Figure 6.2). However, in most intermediates the response curve was linear so that quantitative determination could be carried out by dividing the sample into two and adding a known amount of a standard to one half. This would be internal standardisation very similar to that used in enzymatic assays.

3) Investigations carried out to determine to what extent contamination occurred of tissue extracts by the reagents used in the purification procedures

Because of the nature and the amount of reagents used in the purification of tissue extracts it was of importance to test whether impurities in the reagents contributed to the peaks observed in GLC chromatograms of tissue samples. For instance if an impurity in the ethanol used in the extraction of tissue, passed through the purification procedures, at a level of 5 parts per million this would give a peak larger than most seen from tissue samples.

20 ml of 60% aqueous ethanol was treated as an imaginary tissue extract. It was worked up with 5 other genuine samples so that it would not be given speoial treatment, and using an ion-exchange column which had been previously used for the purification of at least two tissue extracts. This sample after silylation was chromatographed at the highest sensitivity used in the analysis of tissue (attenuation setting x 100). As can be seen in Figure 6.3, the peaks present were very minor, TMS-α- and TMS-β-D-glucose being present. This contamination represents a carry over of less than 0.05%. However, the object of the purification was to reduce the very large amount of glucose present in tissue extracts to as low amounts as possible. The glucose was not being assayed itself. There was no evidence of any other gross interference.

(contd. on p. 172)


A
5·0
4·0
3·0
2·0
1·0
PEAK HEIGHT RATIO (TO DOCOSANE)
0
0·1
0·4
ml added standard
1·0

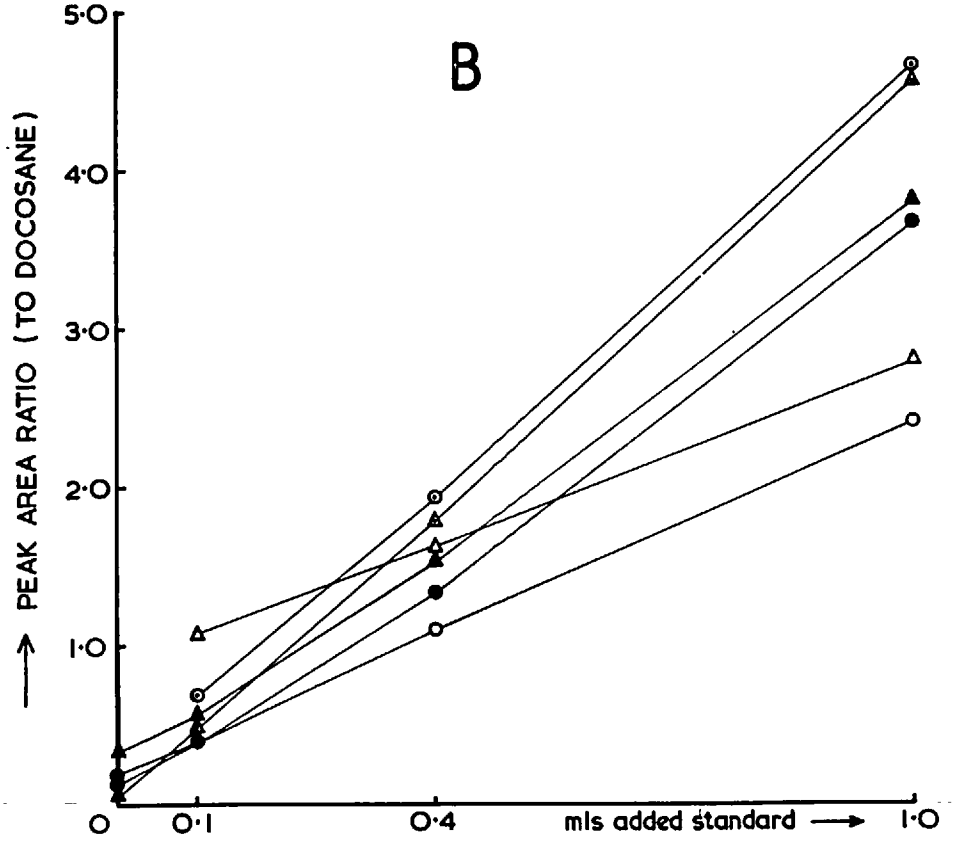

B
5·0
4·0
3·0
2·0
1·0
PEAK AREA RATIO (TO DOCOSANE)
0
0·1
0·4
mls added standard
1·0

Figure 6.1 The effect of increasing the amount of tissue extract on the standard curve

An aqueous ethanolic extract of liver was used. Aliquots corresponding to 5, 20 and 50 mg wet weight of tissue were taken. A standard solution of the intermediates was added to each aliquot (40, 200, 500 nmoles of each). Each was then evaporated to dryness and then treated with 100 µl of BSTFA:pyridine:TMCS (2:1:1 by vol). 5 µl of this reagent was analysed on a 5' x ⅛" column of 3% OV-1 on Gas Chrom Q. Temperature programmed analysis: 100°C for 5 minutes, then 100-250°C at 4°C per minute, was used. Docosane was added (80 nmoles) as internal standard and gave a constant response with changing amounts of tissue extract. Hexadecane also added as an internal standard decreased with decreasing amounts of tissue.

The relative peak area response was obtained by dividing the peak area of the compound by the peak area for docosane. This response was then plotted against the amount of the compound added to the extract. Similarly the relative peak height response was calculated.

Identification:

A.
- △ F6P + 5 mg liver
- ⊙ G6P + 5 mg liver
- ▲ F6P + 20 mg liver
- ● G6P + 20 mg liver
- △ F6P + 50 mg liver
- ○ G6P + 50 mg liver

B.
- △ succinate + 5 mg liver
- ⊙ fumarate + 5 mg liver
- ▲ succinate + 20 mg liver
- ● fumarate + 20 mg liver
- △ succinate + 50 mg liver
- ○ fumarate + 50 mg liver

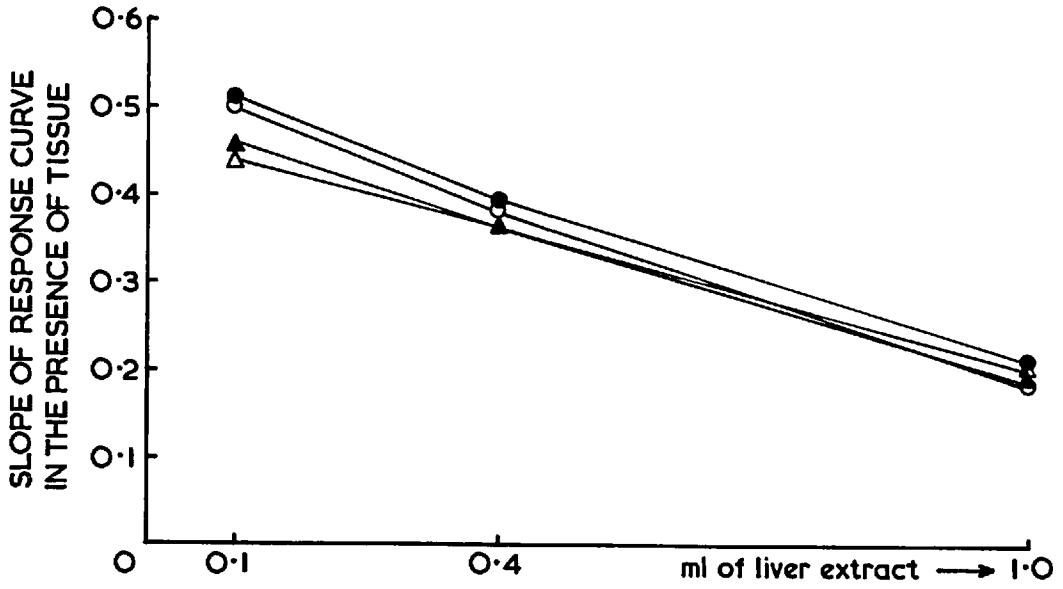

SLOPE OF RESPONSE CURVE IN THE PRESENCE OF TISSUE
0·6
0·5
0·4
0·3
0·2
0·1
0
0·1
0·4
ml of liver extract ⟶
1·0

Figure 6.2 The variation in the slope of the peak area response versus amount of compound added with differing amounts of tissue extract

These results were obtained from the data presented in Figure 6.1. The slope of the response curve was measured and plotted against the amount of liver extract used.

Identification:

- ● F6P
- ○ G6P
- ▲ succinate
- △ fumarate

As mentioned earlier phosphate was a problem but it could be removed by precipitation. The use of deionised distilled water kept contamination during purification down to low values.

4) The removal of amino acids from tissue extracts

It was necessary to remove amino acids from tissue extracts since they would not be silylated at 4°C and would therefore give erroneous recoveries of radioactivity if they were radioactive, and if they were converted to volatile derivatives by suitable heating these might interfere with the analysis. Amino acids eluted from the AG 1 column with 0.5M sodium formate would be retained by the AG 50 column when the sodium formate eluate was passed through. This was checked by passing the twenty common amino acids (1 μmole each) through the purification procedure. Only 5% of the original ninhydrin response remained after elution from the AG 50 column (probably the dicarboxylic amino acids, glutamic and aspartic acids).

In addition, the amino acid mixture was silylated as was the sample taken through purification procedures. GLC analysis indicated that the amino acids did not interfere with the analysis (Figures 6.4A and 6.4B), the peaks at the end of the analysis eluted after TMS-G6P. The extra peaks in the purified sample corresponded to those derived from the purification procedure (Figure 6.3).

5) The method used for the analysis by GLC of intermediates in tissue

A schematic diagram to summarise the isolation and purification step is shown in Figure 6.5. Very rapid freezing of tissue was necessary to obtain the result which reflected the metabolic situation

(contd. on p.176)

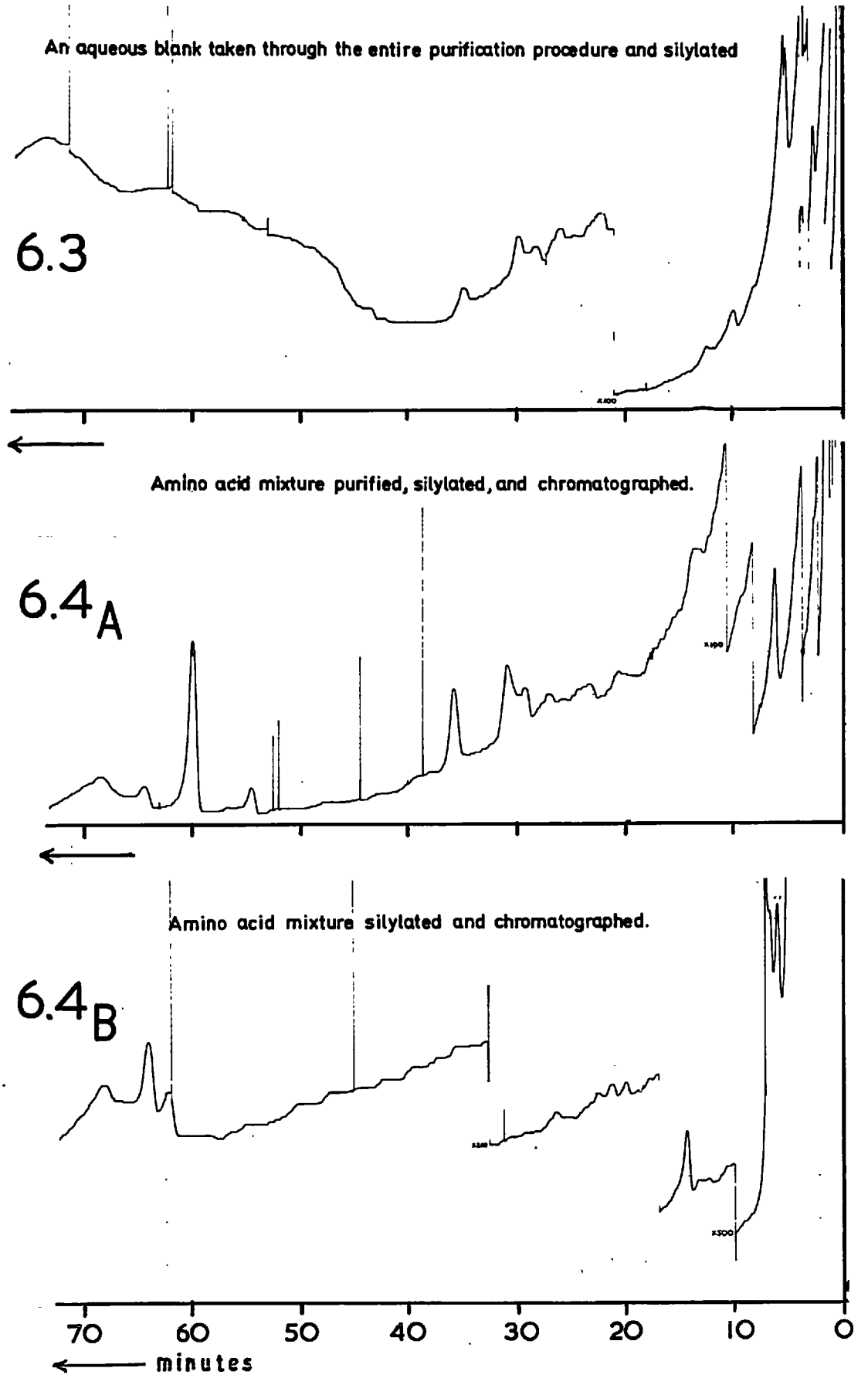

An aqueous blank taken through the entire purification procedure and silylated
6.3
Amino acid mixture purified, silylated, and chromatographed.
6.4A
Amino acid mixture silylated and chromatographed.
6.4B
70
60
50
40
30
20
10
0
minutes

Figure 6.3 An examination of those peaks observed in chromatograms which did not arise from tissue

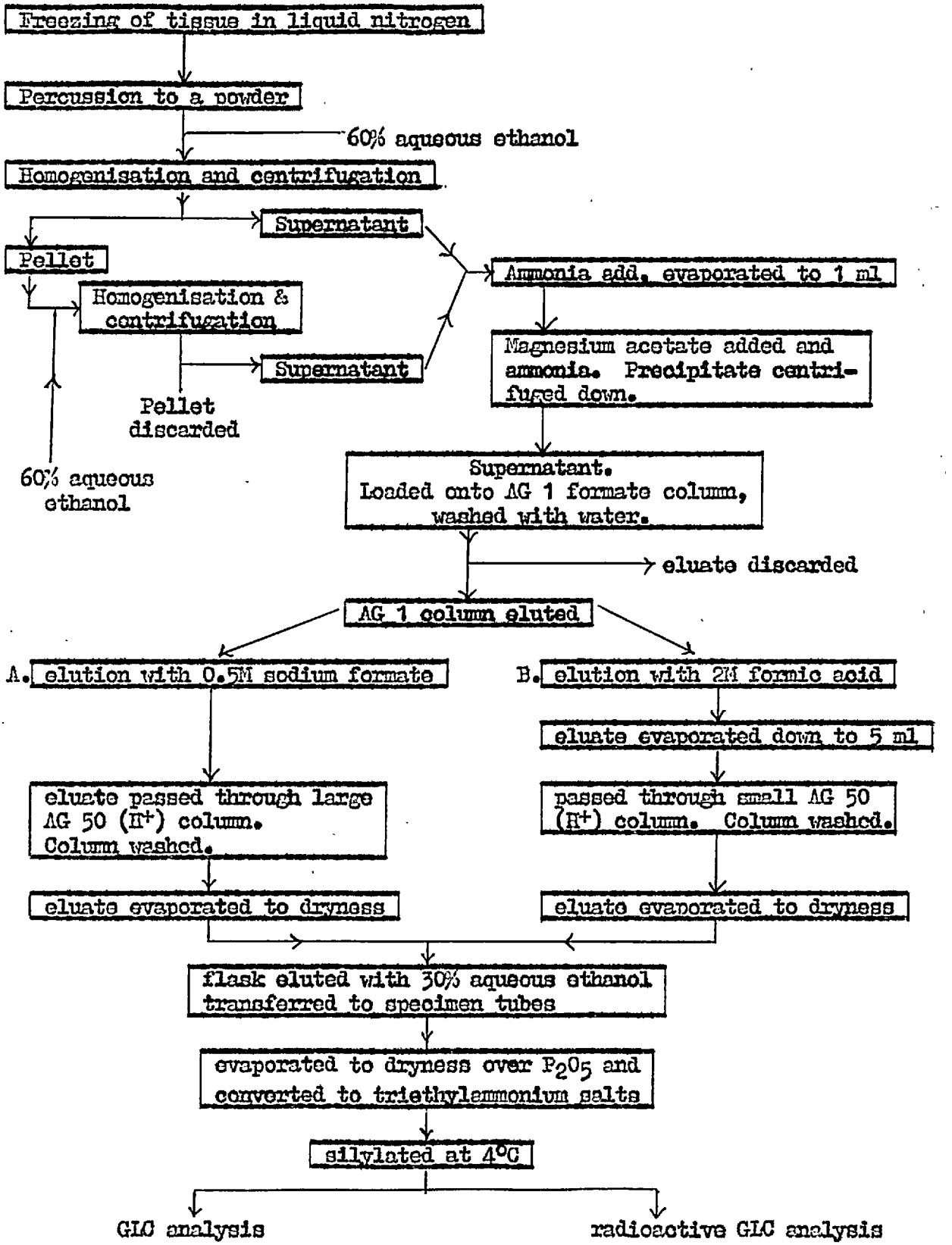
20 ml of 60% aqueous ethanol was treated as an imaginary tissue homogenate. It was evaporated to dryness and the residue dissolved in 10 ml water. This was absorbed onto an AG 1 (formate form) resin column (12 x 0.5 cm), the column being washed with 9 x 10 ml water. The column was eluted with 4 x 10 ml 0.5M sodium formate. This eluate was passed through an AG 50 (H^+ form) resin column (30 x 2.5 cm) washing the column with a further 40 ml water. The combined eluate and washings were evaporated to dryness, and the concentrate transferred to a 75 x 12 mm specimen tube with 3 x 1 ml 33% aqueous ethanol. This was evaporated to dryness and reacted with 0.1 ml of BSTFA:pyridine:TMCS (2:1:1, by vol). 5 µl of this was injected onto a 3% SE-52 on Chromosorb W (AW-DMCS, 80-100 mesh) column (5' x ¼") using the temperature programme 100-250°C at 2°C per minute. The sensitivity of the analysis was the highest used in any experiment (attenuation x 100).

Figure 6.4 Examination of the Interference by peaks derived from amino acids during the gas liquid chromatographic separation of trimethylsilyl derivatives of carbohydrate intermediates from tissue

Figure 6.4A A mixture of the 20 common amino acids (1 µmole each) was evaporated to dryness and reacted with 0.1 ml BSTFA:pyridine:TMCS (2:1:1, by vol) at 4°C. 5 µl were injected onto a 3% SE-52 on Chromosorb W (AW-DMCS, 80-100 mesh) column using the temperature programme 100-250°C at 2°C per minute.

Figure 6.4B A mixture of the above amino acids was absorbed onto an AG 1 (formate form) resin column (12 x 0.5 cm) and the column washed with 10 x 10 ml water. It was then eluted with 4 x 10 ml 0.5M sodium formate. This fraction was passed through an AG 50 (H^+ form) resin column (30 x 2.5 cm), washing the column with 40 ml water. The combined eluate and washing was evaporated to dryness. The concentrate was transferred to a specimen tube and silylated with 0.1 ml BSTFA:pyridine:TMCS (2:1:1, by vol) at 4°C. 5 µl was analysed by gas liquid chromatography as described for Figure 6.4A.

Figure 6.5 Schematic representation of purification and silylation procedures



being studied. For *in vivo* studies the tissue was freeze clamped *in situ* with flat-faced tongs cooled in liquid nitrogen (Wollenberger et al, 1960). For perfusion studies freeze clamping was also used. For tissue suspensions, the suspension was not centrifuged, but poured *in toto* into a beaker containing liquid nitrogen. The frozen tissue was percussed in amounts not exceeding 5 g in a stainless steel mortar cooled in liquid nitrogen. The tissue was then powdered in a pestle and mortar cooled with solid CO_2. The powder was transferred to a cold plastic centrifuge tube (50 ml). Cold (-10°C) 60% aqueous ethanol was added (for up to 2.5 g, 10 ml, and for 2.5-5 g, 4 ml/g). The mixture was homogenised until all the tissue was dispersed, the solvent initially partly froze. The homogenate was allowed to stand at 0°C for ten minutes, its temperature not rising above -10°C. It was then centrifuged (10,000 g for 10 mins) at -5°C and the supernatant removed. The pellet was re-extracted with the same volume of 60% aqueous ethanol and centrifuged.

The two supernatants were combined, and 0.880 ammonium hydroxide added dropwise to pH 8-9. The extract was concentrated to 1 ml under vacuum (water-pump) using a rotary evaporator at 30°C. The concentrate was carefully transferred to a centrifuge tube, and with the washings of the flask made up to 5 ml. Magnesium acetate (30 mg in 5 ml) was added followed by a few drops of ammonium hydroxide to pH 9. The white floco precipitate of magnesium ammonium phosphate was removed by centrifugation (2000 g for 10 minutes) and the supernatant carefully decanted.

It was loaded onto an AG 1 (formate form) resin column (12 x 0.5 cm, 100-200 mesh). When it had all been absorbed onto the

column, the column was washed with a further 9 x 10 ml deionised distilled water stepwise, each 10 ml being run through the column before the next 10 ml was added to the reservoir.

The intermediates retained on the AG 1 column were eluted with 4 x 10 ml 0.5M sodium formate, or with 4 x 10 ml 2M formic acid (an alternative method not used in the applications). The sodium formate eluate was then passed through an AG 50 (H^+ form) resin column (30 x 2.5 cms, 200-400 mesh). The column was washed with 40 ml water. The eluate and washings were combined and evaporated to dryness under vacuum (water pump) using a rotary evaporator at 30°C. The flask was removed from the evaporator as soon as it was seen to be dry. The dry residues were eluted with 3 x 1 ml 33% aqueous ethanol. The eluted extracts were pipetted into a specimen tube (75 x 12 mm). The tube was stored under vacuum over phosphorous pentoxide overnight. The following morning triethylamine (50 µl) was added and the tube dried for a further 4 hours.

The silylation mixture (bis(trimethylsilyl)-trifluoroacetamide: pyridine:trimethylchlorosilane, 2:1:1 by vol) (0.1 ml), was added and the tube sealed, shaken and placed in a refrigerator at 4°C. Samples gave repeatable chromatograms for over two weeks when stored at this temperature. Samples left overnight at 4°C were ready for analysis the following morning.

5 µl aliquots were analysed by gas liquid chromatography. The column packing used was 3% SE-52 on Chromosorb W (AW-DMCS, 80-100 mesh) in 5' x ¼" O.D. glass columns for the radioactive GLC; 3% OV-1 on Gas Chrom Q (100-120 mesh) in a 5' x ¼" column was used for mass analyses. The column packing was deactivated before use by repeated injection of the silylation reagent. The flow rate of the carrier gas, nitrogen, was adjusted to be 60 ml/min in the analytical and reference columns. A

temperature programmed analysis was used, 100-250°C at 2°C per minute for the radioactive GLC, and 100°C for 5 minutes, then 100-250°C at 4°C per minute for the mass analyses. When analyses were not being carried out the temperature of oven was maintained at 250°C. Before use the oven was cooled to 60°C and then heated to 100°C and left to equilibrate for 15 minutes. Detection of the eluted compounds was carried out with a flame ionisation detector (with reference detector for backing off) in a separate oven maintained at 250°C. The hydrogen flow rate in each detector was 80 ml/minute and the air flow rate 250 ml/minute. The amplified detector output was recorded in parallel on a Kent Mark III recorder (10 mV) and Rikadenki B-341 three channel recorder (10 mV). The Kent recorder was used to retransmit the analogue signal to an analogue to digital convertor for encoding and recording onto punched tape.

For analyses involving radioactive compounds a stainless steel splitter (1:1) was fitted to the end of the glass column. The vapours diverted to the scintillation cell were condensed into the scintillation fluid, and the radioactivity present monitored using a Packard model 3022 spectrometer. The output of this instrument was recorded on Channel 2 of the Rikadenki recorder. Channel 3 of this recorder was used to check the temperature of the heating line (250°C).

Determinations of the mass of peaks present were made by comparing the areas of each peak in the unknown mixture and the areas of each peak in a standard mixture which was worked up in parallel with the unknown. The increase in radioactivity in the scintillation cell as each compound was eluted (corrected for the split ratio and efficiency of counting, F = 3.64) was divided by the mass to obtain the specific radioactivity of each compound.

CHAPTER 7

SOME SIMPLE APPLICATIONS OF THE METHODS DEVELOPED FOR THE STUDY OF CARBOHYDRATE INTERMEDIATES IN TISSUE

Three situations have been studied. Firstly, the effect of streptozotocin induced diabetes on the levels of intermediates in adult rat heart has been examined. Secondly, the effect of anoxia on the carbohydrate intermediates and the metabolism of [U-^{14}C]-glucose in rat heart has been studied. Finally the metabolism of [1-^{14}C]-glucose and [6-^{14}C]-glucose by P.polycephalum plasmodia, an organism strongly suspected of having a pentose phosphate shunt pathway (see Appendix 1), has been studied.

1) The effect of streptozotocin induced diabetes on the in vivo concentrations of carbohydrate intermediates of rat heart

A diabetic state, i.e. blood glucose greater than 300 mg%, was induced in a 250 g male rat by the injection of streptozotocin (16 mg) in saline (Rakieten et al, 1963) via the tail vein. After 48 hours this animal and an untreated control rat from the same group were ether anaesthesized, their chests were opened and their hearts freeze clamped *in situ* with tongs cooled in liquid nitrogen (Wollenberger et al, 1960). Obviously blood was not washed out of the hearts before clamping so that some contamination did occur. The hearts were treated as described in the Method in Chapter 6. GLC analysis was carried out on 3% SE-52 on Chromosorb W (AW-DMCS, 80-100 mesh) column packing (see Figure 7.1 and Table 7.1).

Certain gross effects were obvious; G6P and citrate were both elevated and ∝-glycerophosphate and malate were reduced in the diabetic rat heart. Similar trends have been observed in alloxan induced diabetic rat hearts (Newsholme & Randle, 1964; Garland & Randle, 1964), but these hearts were perfused *in vitro* so that the absolute figures may well be different.

2) The effect of reduced oxygen tension on the concentration of carbohydrate intermediates in the isolated perfused rat heart

Hearts of 250-300 g rats were excised and perfused according to the methods of Chain et al (1970). In each experiment the heart was perfused in Krebs-Henseleit (1932) bicarbonate buffer (50 ml) containing glucose (11 mM). For high potassium buffer the KCl concentration was raised from 5 mM to 15 mM and to maintain an ionic balance the NaCl concentration was lowered from 118 mM to 108 mM. The heart was preperfused for 15 minutes and then perfused for 30 minutes with fresh buffer containing 125 uCi [U-^{14}C]-glucose (purified by passage through AG 1 resin (formate form) just before the start of the experiment). The buffer was recirculated and continuously gassed with either 95% O_2/5% CO_2 or 95% N_2/ 5% CO_2. The non-working Langendorff heart preparation was used for each experiment. At the end of the perfusion period the hearts were washed through with ice-cold saline and then rapidly freeze clamped with tongs cooled in liquid nitrogen (Wollenberger et al, 1960).

The results of the experiments carried out are summarised in Table 7.2. Both lactate and ∝-glycerophosphate had increased specific radioactivities when the hearts were anoxic. Curiously no radioactivity was associated with TMS-G6P despite it being present in

(contd. on p.184)

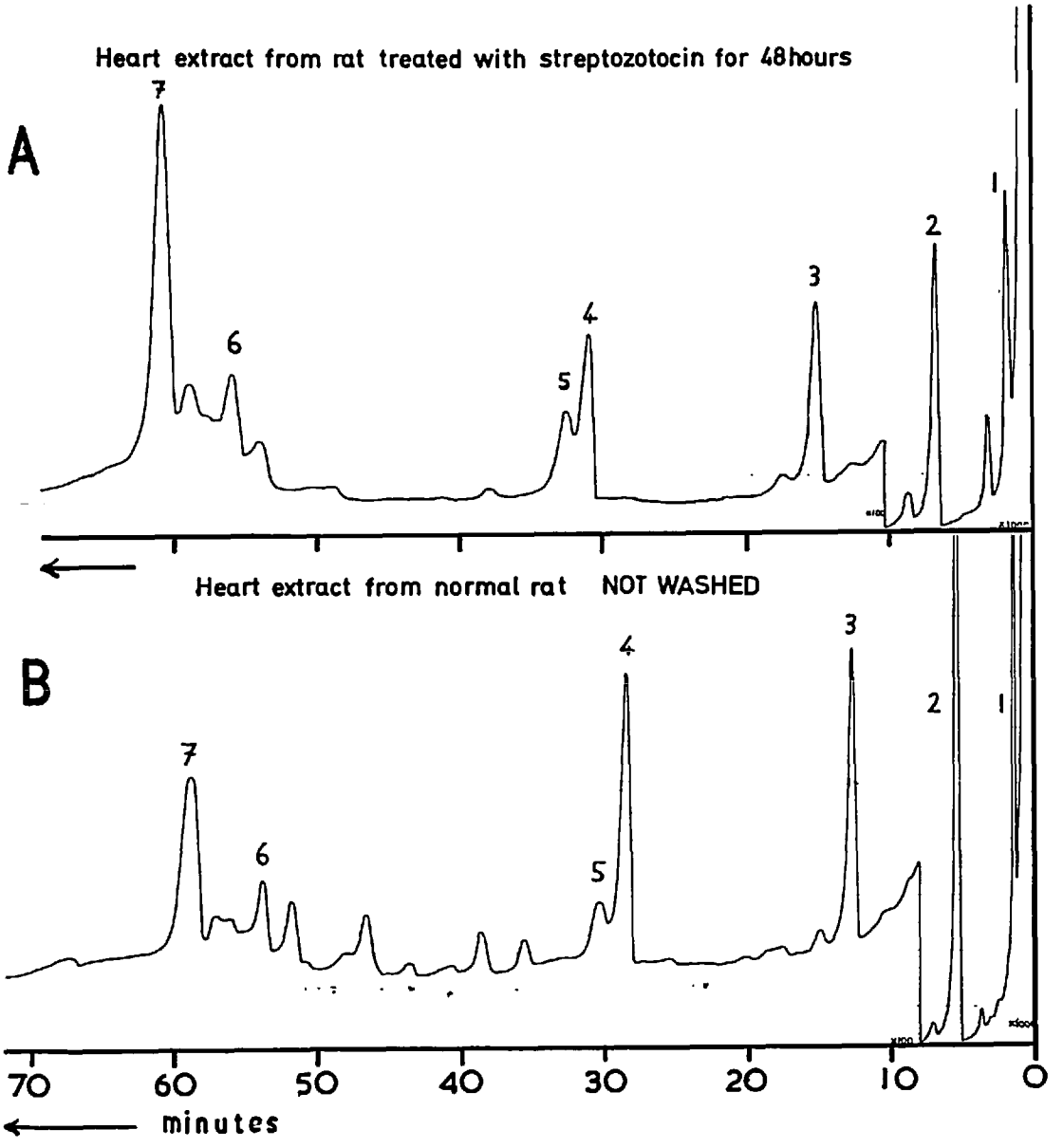

Heart extract from rat treated with streptozotocin for 48hours
A
7
6
5
4
3
2
1
Heart extract from normal rat NOT WASHED
B
7
6
5
4
3
2
1
70
60
50
40
30
20
10
0
minutes

Figure 7.1 The effect of streptozotocin induced diabetes on the in vivo levels of carbohydrate intermediates in rat heart.

Both hearts were freeze clamped *in situ* (Wollenberger et al, 1960) after opening the chests of the animals under ether anaesthesia. The frozen hearts were extracted and the extract worked up as described in the Method in Chapter 6. 5 µl aliquots were analysed on 3% SE-52 on Chromosorb W (AW-DMCS, 80-100 mesh) using the programme 110-230°C at 2°C per minute.

Peak identification:

1. lactate 2. phosphate 3. malate 4. ∝-glycerophosphate
5. citrate 6. fructose-6-phosphate 7. glucose-6-phosphate

Chromatogram A

Rat treated with streptozotocin 48 hours previously.

Chromatogram B

Normal rat (250 g).

Table 7.1

The levels of G6P, F6P, ∝-glycerophosphate, citrate and malate in hearts from the normal fed rat and the streptozocin induced diabetic rat as determined by the GLC method

The hearts were obtained as described in the text and then purified as described in the Method in Chapter 6. Gas chromatographic analysis was carried out on 3% SE-52 on Chromosorb W (AW-DMCS, 80-100 mesh) as described in the legend to Figure 7.1. The results are expressed as μmoles/100 g wet weight of tissue. They were obtained from the chromatograms in Figure 7.1.

Compound	Normal Fed Rat	Streptozotocin Diabetic Rat
G6P	33	63
F6P	7	11
∝-glycerophosphate	27	16
citrate	12	20
malate	22	14

large amounts in the aerobic heart (see Figure 7.2). Reduced oxygen tension lowered the G6P level considerably. High K^+ concentration although stopping the heart beating, and reducing the glucose uptake and lactate production into the medium, did not alter the intracellular levels or specific radioactivities of lactate or ∝-glycerophosphate.

3) The distribution of the ^{14}C-label in glucose metabolites from [1-^{14}C]-glucose and [6-^{14}C]-glucose after incubation with the plasmodia of P.polycephalum

The myxomycete, Physarum polycephalum has only been recently pure cultured and very little if any is known about its carbohydrate metabolism. In Appendix 1 some preliminary experiments to determine the nature of its carbohydrate metabolism were carried out.

Evidence is presented there indicating that [1-^{14}C]-glucose was oxidised to $^{14}CO_2$ more rapidly than [6-^{14}C]-glucose by the plasmodia of P.polycephalum. This was demonstrated by a positive effect (more ^{14}C-label in CO_2 from [1-^{14}C]-glucose than [6-^{14}C]-glucose) and a negative effect, less ^{14}C-label in lipid after incubation with [1-^{14}C]-glucose than [6-^{14}C]-glucose). Both these observations indicated that the pentose phosphate shunt was operating during aerobic incubation of these plasmodia. If this was so then it would be expected that certain intermediates would have different specific radioactivities, depending on whether the glucose was labelled with ^{14}carbon on the C-1 or C-6 position. In fact any intermediate after 6-phosphogluconate in the metabolic sequence should show a lower specific radioactivity from 1-^{14}C -glucose than from [6-^{14}C]-glucose because of the $^{14}CO_2$ lost going from 6-phosphogluconate to ribulose-5-phosphate.

(contd. on p.188)

Table 7.2

Effect of reduced oxygen tension and high potassium concentration on certain metabolic parameters in the perfused isolated rat heart

Hearts from 250-300 g rats were perfused according to the methods described in the text. Each was perfused in buffer containing 11 mM glucose (0.19 μCi/μmole). Hearts were perfused in the following manner:

1. aerobic preperfusion (15 minutes), aerobic perfusion (30 minutes) active heart.
2. aerobic preperfusion (15 minutes), anoxic perfusion (30 minutes) heart activity declined.
3. anoxic preperfusion (15 minutes), anoxic perfusion (30 minutes) heart activity low.
4. anoxic preperfusion (15 minutes), anoxic perfusion in high K^+ (30 minutes) heart activity ceased.

After freeze clamping of each heart (Wollenberger et al, 1960) the matabolic parameters in the table below were determined. The extracellular glucose and lactate concentrations were determined enzymatically by Miss P. Owen. The intracellular lactate and ∝-glycerophosphate specific radioactivities and concentrations were determined by the radioactive GLC Method detailed in Chapter 6. The radioactivity associated with the amino acids was that which was eluted with ammonium hydroxide from the AG 50 resin column.

\+ specific radioactivity units μCi/μmole
* concentration units μmole/g

HEART	EXTRACELLULAR		INTRACELLULAR LACTATE		INTRACELLULAR ∝-GP		RADIOACTIVITY IN AMINO ACIDS (μCi)
	Glucose Uptake umoles	Lactate Output umoles	Specific Activity +	Concentration *	Specific Activity +	Concentration *	
1	30	30	0.009	2.5	0.007	0.4	0.17
2	115	125	0.015	3.0	0.018	1.8	0.21
3	115	135	0.023	2.2	0.022	2.2	0.07
4	20	65	0.023	2.3	0.019	2.1	0.07

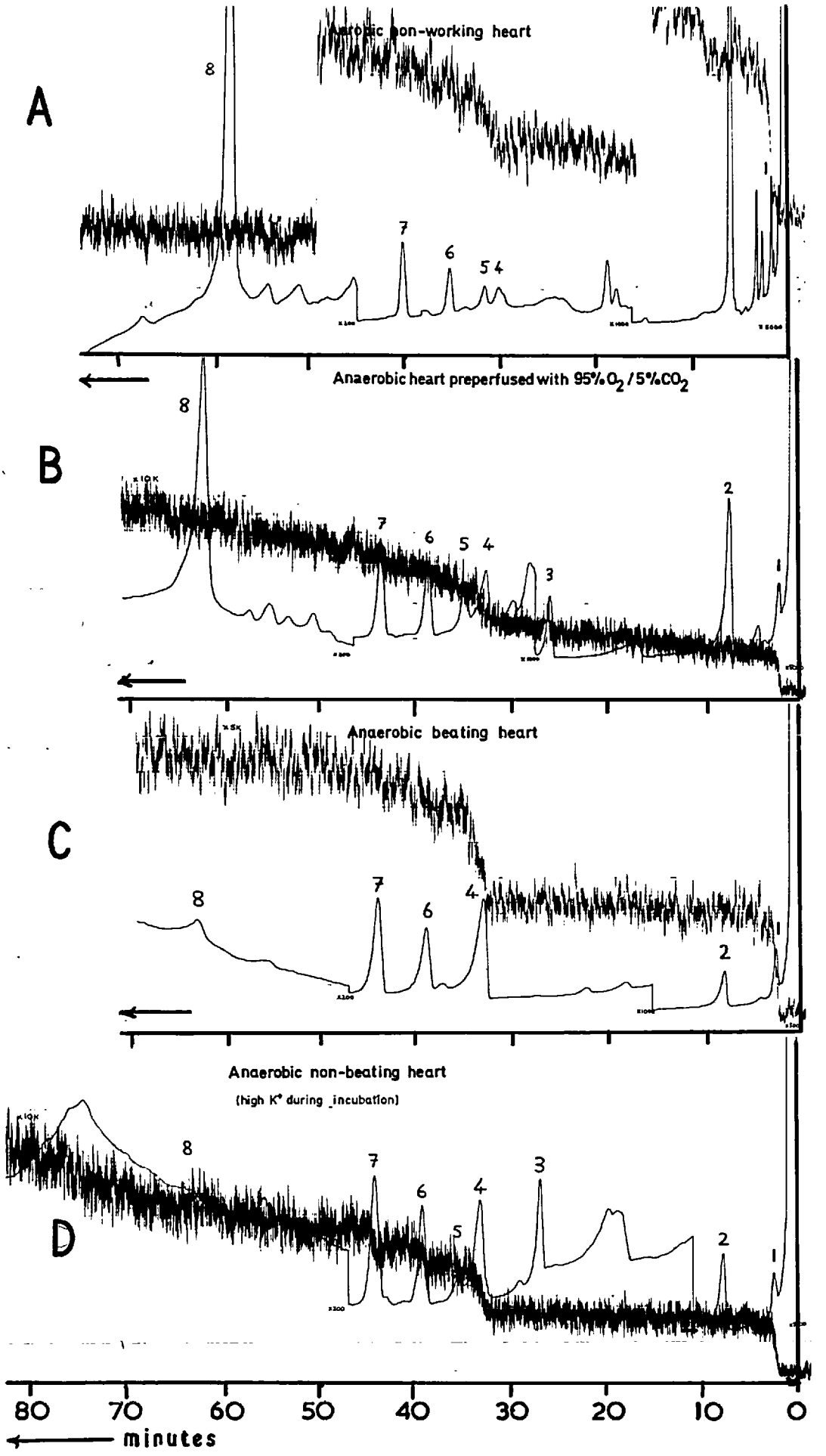

A
Aerobic non-working heart
8
7
6
5 4
1
Anaerobic heart preperfused with 95% O_2 / 5% CO_2
B
8
7
6
5
4
3
2
1
Anaerobic beating heart
C
8
7
6
4
2
Anaerobic non-beating heart
(high K^+ during incubation)
D
8
7
6
4
5
3
2
1
80
70
60
50
40
30
20
10
0
minutes

Figure 7.2 The effect of reduced oxygen tension and high K^+ concentration during incubation with [U-^{14}C]-glucose on the carbohydrate intermediates in perfused isolated rat heart

Each heart was perfused with Krebs Henseleit (1932) buffer containing 11 mM glucose (specific radioactivity 0.19 μCi/μmole) for 30 minutes after a preperfusion period of 15 minutes with the same buffer containing no radioactivity. At the end of the 30 minute period the hearts were washed through with 20 ml ice-cold saline, and then freeze clamped with tongs cooled in liquid nitrogen (Wollenberger et al, 1960). The heart was extracted, purified and silylated as described in the Method in Chapter 6. GLC analysis was carried on 3% SE-52 on Chromosorb W (AW-DMCS, 80-100 mesh).

Peak identification for each chromatogram:

1. lactate 2. phosphate 3. unknown 4. α-glycerophosphate
5. citrate 6. α-D-glucose 7. β-D-glucose 8. glucose-6-phosphate

Chromatogram A

Heart preperfused with 95% O_2/5% CO_2 and perfused with the same gas.

Chromatogram B

Heart preperfused with 95% O_2/5% CO_2, perfused with 95% N_2/5% CO_2.

Chromatogram C

Heart preperfused with 95% N_2/5% CO_2 and perfused with the same gas.

Chromatogram D

Heart preperfused with 95% N_2/5% CO_2 and perfused with the same gas, but in high K^+ concentration (15 mM) buffer.

An experiment was set up to study the movement of the ^{14}C-label from [1-^{14}C]-glucose and [6-^{14}C]-glucose through glycolysis to the Krebs cycle when metabolised by the plasmodia of P.polycephalum.

Plasmodia from the active phase of growth were incubated in standard medium less glucose and peptone. Glucose was added to give a concentration of 1 mM and the plasmodial suspension was perfused at 24°C with 95% O_2/5% CO_2 for 5 minutes. Then [1-^{14}C]-glucose (to give a specific radioactivity of 0.75 μCi/μmole) was added. Aliquots of the plasmodial suspension were removed at 1 minute and 6 minutes, and were immediately frozen in liquid nitrogen. An identical incubation was performed in parallel adding [6-^{14}C]-glucose (to give a specific radioactivity of 1.50 μCi/μmole).

The frozen samples (also containing metabolites from the media) were treated as described in the Method in Chapter 6. Radioactive GLC on the silylated samples was carried out using 3% SE-52 on Chromosorb W (AW-DMCS, 80-100 mesh).

The radiochromatograms so obtained are shown in Figure 7.3. As could be seen no radioactivity was associated with lactate, malate, fumarate, succinate or citrate after incubation for 1 minute. However, after six minutes radioactivity could clearly be seen with lactate, malate and citrate after incubation with [6-^{14}C]-glucose. Radioactivity may well also have been associated with fumarate and succinate, but this was in doubt because TMS-fumarate and TMS-succinate were eluted on the trailing side of the large TMS-phosphate peak. In contrast only a very small amount of radioactivity was associated with these compounds after incubation with [1-^{14}C]-glucose. This was further evidence for the existence of the pentose phosphate pathway. The specific radioactivities for G6P, F6P and malate (see Table 7.3) show this very clearly as well.

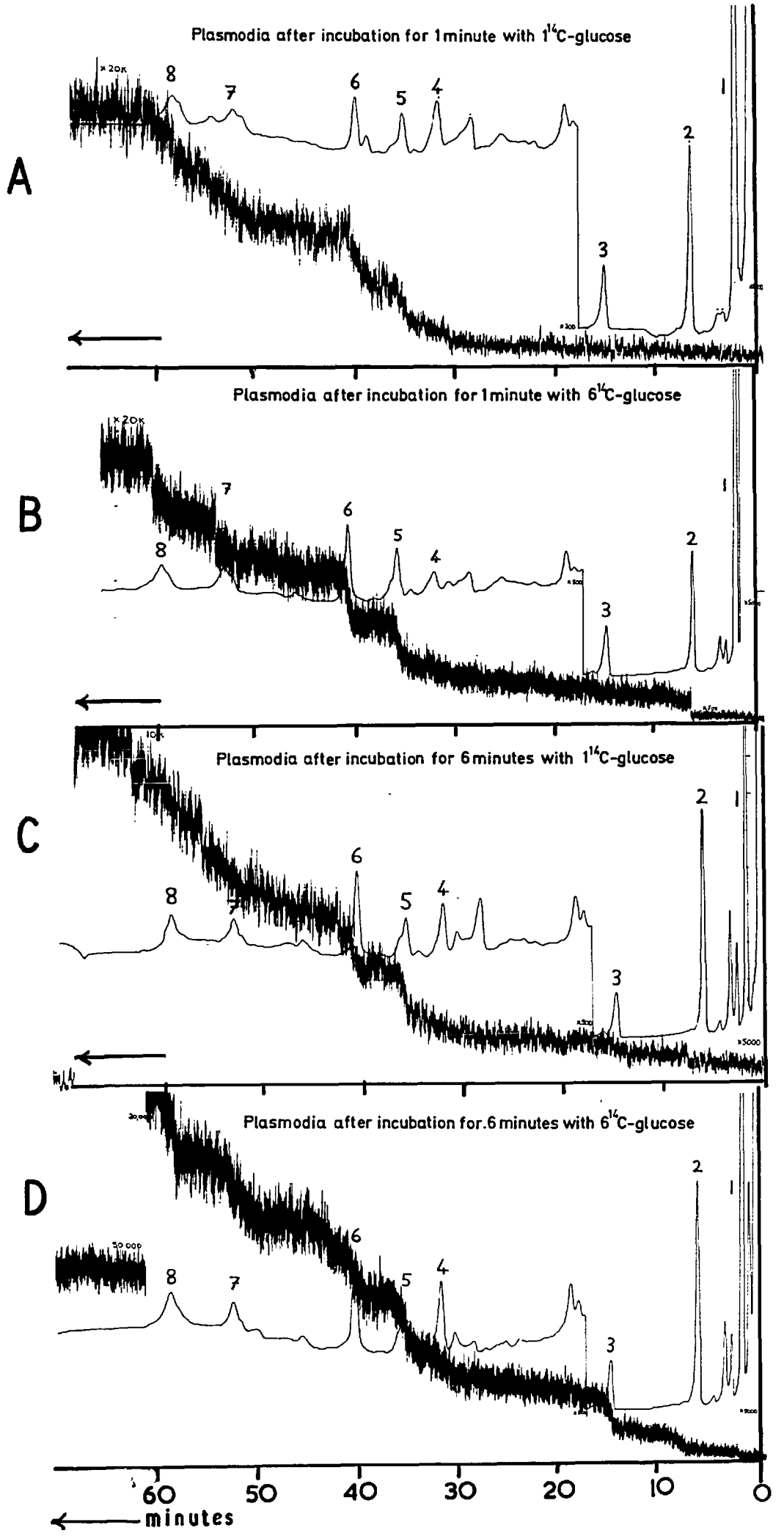

A
Plasmodia after incubation for 1 minute with 1^{14}C-glucose
B
Plasmodia after incubation for 1 minute with 6^{14}C-glucose
C
Plasmodia after incubation for 6 minutes with 1^{14}C-glucose
D
Plasmodia after incubation for 6 minutes with 6^{14}C-glucose
1
2
3
4
5
6
7
8
60
50
40
30
20
10
0
minutes

Figure 7.3 The distribution of ^{14}C-label in carbohydrate intermediates from [1-^{14}C]-glucose and [6-^{14}C]-glucose after incubation with the plasmodia of P.polycephalum

Plasmodia from the active phase of growth were incubated in standard medium lacking peptone and glucose. Glucose was added (1 mM) and the medium was perfused at 24°C with 95% O_2/5% CO_2. After 5 minutes incubation [1-^{14}C]-glucose (to give specific radioactivity 0.75 μCi/μmole) and [6-^{14}C]-glucose (to give specific radioactivity 1.50 μCi/μmole) were added to separate but similar flasks. Aliquots taken for assay were frozen immediately in a beaker of liquid nitrogen. The extraction, purification and subsequent radioactive GLC assay were as described in the Method in Chapter 6.

Peak identification:

1. lactate 2. phosphate 3. malate 4. citrate
5. α-D-glucose 6. β-D-glucose 7. fructose-6-phosphate
8. glucose-6-phosphate

Chromatogram A

Aliquot of plasmodia removed after 1 minute incubation with [1-^{14}C]-glucose.

Chromatogram B

Aliquot of plasmodia removed after 1 minute incubation with [6-^{14}C]-glucose.

Chromatogram C

Aliquot of plasmodia removed after 6 minutes incubation with [1-^{14}C]-glucose.

Chromatogram D

Aliquot of plasmodia removed after 6 minutes incubation with [6-^{14}C]-glucose.

Table 7.3

The specific radioactivities* of G6P, F6P and malate after incubation of P.polycephalum plasmodia with [1-^{14}C]-glucose and [6-^{14}C]-glucose after 1 minute and 6 minutes

The measurements of specific radioactivity were obtained using the radioactive gas liquid chromatography technique described in Chapter 6. Plasmodia of P.polycephalum were incubated in standard medium lacking peptone, and at a glucose concentration of 1 mM. [1-^{14}C]-glucose (to give specific radioactivity 0.75 µCi/µmole) and [6-^{14}C]-glucose (to give specific radioactivity 1.50 µCi/µmole) were added and aliquots of the plasmodial suspension removed at 1 minute and 6 minutes and immediately frozen in liquid nitrogen.

	[1-^{14}C]-glucose		[6-^{14}C]-glucose	
	1 min	6 mins	1 min	6 mins
G6P	0.28	0.40	0.65	1.14
F6P	0.14	0.18	0.38	0.50
Malate	0.00	< 0.01	0.00	0.04

* units of specific activity µCi/µmole

CHAPTER 8

DISCUSSION

1) The preparation of volatile derivatives for gas chromatography

a) The dimethylester-trimethylsilyl ether derivatives of sugar phosphates

The work on these derivatives of the sugar phosphates, G6P, F6P and G1P, showed clearly that they were not suitable for quantitative measurements in tissue. The work of Wells et al (1964) who introduced these derivatives has been quoted (Hancock, 1968) as being a means by which sugar phosphates could be analysed. However, the problems of reproducibility proved to be insurmountable (C.C. Sweeley & W.W. Wells - personal communications).

G6P could be reproducibly analysed from aqueous solution (Table 3.3, p. 75), but this was far removed from tissue. Even for G6P there was a side reaction; diazomethane could react with the unblocked C-1 position to give α- and β-methyl glucoside-6-dimethyl phosphate in the same way that α-methylglucoside could be prepared from α-glucose (Kuhn & Baer, 1956). The shoulder preceding the major peaks of the G6P derivative on GLC was due to TMS-α- or β-methylglucoside-6-dimethyl phosphate. The two spots obtained by TLC of methylated G6P (Figure 3.1, p. 64) if eluted separately and then silylated give rise to the two major peaks (most probably the α- and β- forms) and the shoulder respectively.

The derivative for F6P was not even formed reproducibly from an aqueous solution. The problem seemed to lie with the methylation step of the reaction. The silylation step was reproducible (Table 3.2, p. 74). Although only one peak could be observed during GLC analysis

it was likely that it was an occlusion of several peaks. TLC analysis of methylated F6P which gave many spots would support this.

Attempts to form the derivative of G1P were not successful. The use of methanol which reduced the number of protons present so that the free acid of G1P could be handled without hydrolysis (see Figure 3.5, p. 79) did not lead to a suitable derivative. Breakdown to glucose occurred although the amount formed varied according to the method of analysis. TLC indicated less than 20% of the theoretical amount, whereas GLC gave results from 21.9% to 34.0% and enzymatic analysis with glucose oxidase results from 41.4% to 65.8%. It was possible that hydrolysis occurred in the pH5 acetate buffer used for the enzyme analysis.

Mass spectral analysis was not conclusive as to whether trimethyl phosphate was present after methylation of G1P with diazomethane (Figure 3.9, p. 91 and Table 3.6, p. 92). Even if it was present, it could be that this was due to impurity in the G1P, or to inorganic phosphate liberated from G1P free acid in methanol, albeit that those would be small quantities.

b) The trimethylsilyl ester-trimethylsilyl ether derivatives

i) Phosphate containing compounds

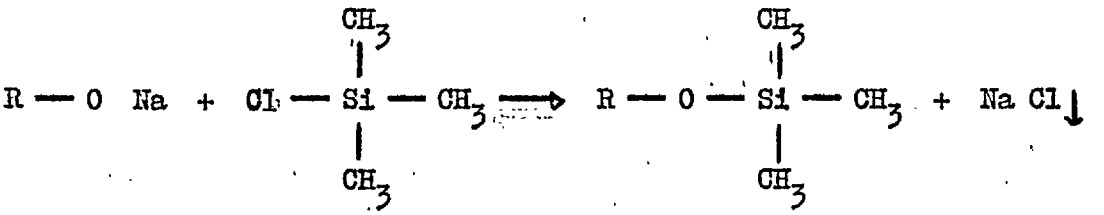
These derivatives first described by Hashizume & Sasaki (1966) were turned to after the failure of the methyl ester-trimethylsilyl ether derivatives. The method for silylation used by these authors, refluxing the compound to be silylated in anhydrous conditions with pyridine:HMDS:TMCS, would not be convenient for routine analyses.

The silyl amide, bis(trimethylsilyl)-acetamide was found to convert compounds to their TMS-derivatives at room temperature, or lower temperature (see Chapter 3.B, p. 93). Since BSA was a good solvent it could be used on its own which meant that silylations could be carried out on a smaller scale. This reagent was also used for the silylation of G1P and G6P by M.G. Horning et al (1967). For the derivative of G1P this was not eluted until 270°C on OV-17 according to M.G. Horning et al (1967). However, in this study, this derivative was eluted rapidly at 200-210°C on OV-17 with a retention time almost identical with TMS-F6P (see Figure 3.10, p. 97). It would seem very unlikely that the peak reported for G1P by M.G. Horning et al (1967) was a derivative of G1P since it was eluted so far after TMS-G6P.

Treatment of G1P with HMDS and TMCS in pyridine led to multiple products of short retention time (see Figure 3.11, p.100). Eisenberg & Bolden (1969) reported that TMS-phosphate was present, but could not identify TMS-α- or -β-glucose. They suggested that on-column pyrolysis was occurring, however, the compounds which might be formed in these circumstances, for example, a 1, 6-anhydroglucan or a glycoceen, were not present. The cause of this breakdown was almost certainly the presence of TMCS in the silylating mixture. TMS-G1P could be prepared with BSA in alkaline conditions to give a single peak (see Figure 3.11b, p.100). The subsequent addition of TMCS to this silylation mixture resulted in the almost complete disappearance of the peak for TMS-G1P and the appearance of peaks of much shorter retention time. Since TMCS generated HCl when it reacted with active hydrogen atoms it was quite likely that the TMS-G1P derivative was unstable in acid conditions. Whether decomposition

occurred on-column or in the reaction tube was not clear, but the broad peak seen (Figure 3.11c, p.100) might well be due to on-column decomposition throughout the column.

Sherman et al (1969) extended the compounds (sugar phosphates) which could be converted to TMS-derivatives by using the mixed reagent BSA:pyridine:TMCS (5:3:2 by vol). With this mixture sodium salts could be silylated as well as the ammonium salts and the triethylammonium salts, but not barium salts (Sherman et al, 1971).

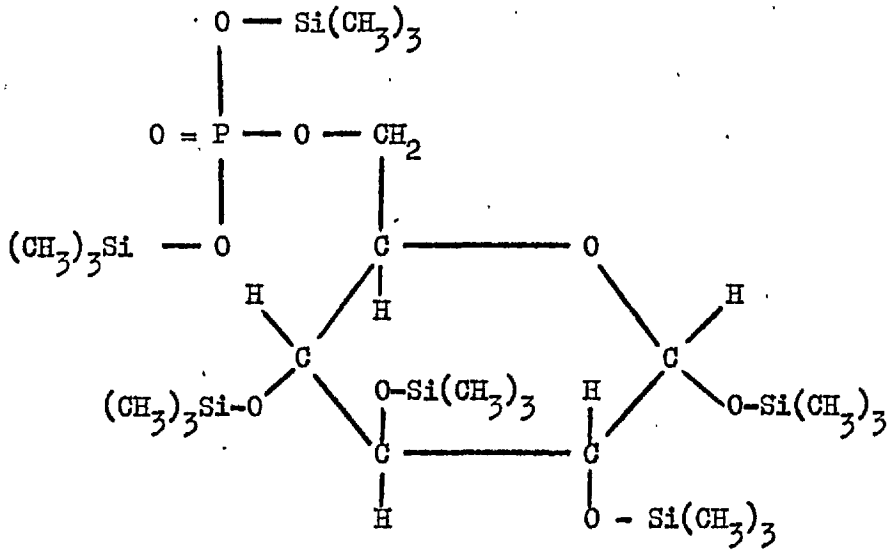


This type of mixed reagent was used in this study as E.C. Horning et al (1967) reported that a mixed reagent of BSA and TMCS was more efficient for silylating steroids, particularly sterically hindered groups. Other ratios of the silylation reagents were used, less BSA could be used with pure solids, but this reflected the increased contaminations one was faced with when studying samples which need some degree of purification (see Chapter 5).

Gas chromatographic analysis of TMS-sugar phosphates has been carried out on DC-710 silicone fluid, JXR, SE-30, OV-1, OV-17 and OV-25 liquid phases (Sherman et al, 1971). In this study no peaks were obtained when using 3% OV-25 on Chromosorb W packing or on polyester phases; peaks were obtained on SE-30, SE-52, OV-1 and OV-17. The retention data on OV-17 for TMS-F6P relative to TMS-α-G6P was similar to that reported elsewhere (0.54 in this study at 210°C,

0.52 at 178°C for Sherman et al, 1971). Similarly for TMS-ribose-5-phosphate the relative retention time was 0.38 in this study against 0.35 for Sherman et al (1971).

Sherman et al (1969) and Zinbo & Sherman (1970) have confirmed that the peaks seen for TMS-sugar phosphates were simply the fully trimethylsilylated sugar phosphate. For G6P they found the mass ion to have a m/e of 692 consistent with tetra 1,2,3,4-O-tetra (trimethylsilyl)-glucose-6-bis(trimethylsilyl)phosphate.



Mass spectral confirmation of the structure of all peaks seen in chromatograms from samples of undefined origin, i.e. from tissue, is essential if

correct interpretation of the qualitative and quantitative data is to be made. This was not done in this study because of a lack of the necessary equipment to be able to pass the compound being eluted from the GLC column into the mass spectrometer successfully.

Complete formation of the TMS-derivative was found to have occurred after several hours at room temperature in BSA (MG. Horning et al, 1967) and after 30 minutes to 3 hours in the silylating reagents (Sherman et al, 1969, 1971). In this study such formation occurred at 4°C overnight. This was sufficient in this study since the major part of the day was spent preparing samples and analysing from previous days. The completeness of the silylation step was indicated indirectly by the recovery of ^{14}C-labelled compounds through the GLC column (see Tables 3.9, p.104 and 3.10, p.105). The data presented showed recoveries greater than 90% for glucose, citric acid, F6P and G6P. This data was in contrast to the data of Jansen & Baglan (1968) who reported recoveries of 30% for TMS-glucose. This marked difference was not likely to be due to their collection procedure, since crude collection of the eluted chromatographic vapours where 70% of the solvent was lost during trapping did not lead to significantly large decreases in recovery (Table 3.9, p.104). The most likely explanation would seem to be that they used a fresh column for each series of analyses. Such columns are prone to completely adsorbing a sample until 'active sites' in the column are saturated. Such phenomena have also been reported by Sherman et al (1971) and as they found the extent to which this occurs can vary considerably between batches of the same column packing. Since Jansen & Baglan (1968) used a Chromosorb G support which was not deactivated, there is a strong possibility that adsorption onto the

column was occurring. Thus they would be correct to report that decomposition of the derivatives occurred in their columns, but it was not true of all column materials and their problem may have been resolved by changing to a more 'inert' support.

ii) Non-phosphate derivatives

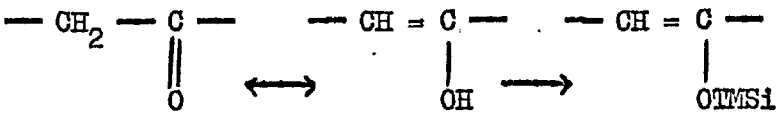
The derivatives of the organic acids, lactate, pyruvate and those of the Krebs cycle, were readily prepared using hexamethyldisilazane and trimethylchlorosilane in pyridine (as for Horii et al, 1965 and Dalgliesh et al, 1966) and using the mixture BSTFA:TMCS:pyridine. BSTFA:pyridine was used by Horning & Horning (1970) to form derivatives of Krebs cycle intermediates in serum.

The oximes of the α-keto acids were prepared for pyruvate, oxaloacetate and α-ketoglutarate, and then were converted to the TMS-derivatives as described by Horii et al (1965).

e.g. $CH_3 - \underset{\underset{O}{\|}}{C} - CO_2H \longrightarrow CH_3 - \underset{\underset{NOH}{\|}}{C} - CO_2H \longrightarrow CH_3 - \underset{\underset{NOTMS}{\|}}{C} - CO_2TMS$

However, these derivatives were not practicable since the excess hydroxylamine used to form them would also react with the silylation reagent, thus increasing the volume required for satisfactory formation of derivatives. The methoximes were prepared in a similar manner. These have the advantage that the excess methoxylamine does not react with the silylation reagents. Treatment of keto-groups with mexthoxylamine was introduced by Fales & Luukkainen (1965) for steroids. Horning et al (1968) and Horning & Horning (1970) have described the use of methoxylamine to stabilise the α-keto acids. Without the

formation of the methoxime a keto-enol transformation can occur



The enol-ether can very readily give up the TMS group which leads to losses on the column. Quinoxalones of pyruvic acid and ∝-keto glutaric acid which were then silylated were used by Hofmann & Killinger (1969), but these derivatives were comparatively involatile and also retained the keto group.

Similar problems of keto-enol transformations occur with the methyl esters of ∝-keto acids (Dalgliesh et al, 1966; Simmonds et al, 1967) and the methyl esters-trimethylsilyl ethers (Dalgliesh et al, 1966). The methyl esters have been used widely for the measurement of Krebs cycle intermediates in plant tissues (Kellog et al, 1964; Rumsey et al, 1964; Canvin, 1965) where the levels are higher, but where the levels are low considerable losses occur after derivatisation making it necessary to use a radioactive recovery determination on every compound (Barnett et al, 1968). Since methyl lactate has a boiling point below that of the solvent chloroform which was concentrated down from 20 ml to 100 ul prior to chromatography, it is a wonder that any was left. This method was briefly investigated with the plasmodia of P.polycephalum (see Appendix 1, Figure A.6, p.285). It was not taken any further since it was later found that carbohydrate intermediates could be studied in a single run using the trimethylsilyl derivatives.

2) Chromatographic separation of TMS-derivatives and production of quantitative data

Gas chromatographic analyses of the TMS sugar phosphates reported in the literature (Hashizume & Sasaki, 1966; Eisenberg & Bolden, 1969; Sherman et al, 1969, 1971) have been mainly carried out isothermally; M.G. Horning et al (1967) used temperature programmed analysis for the separation of TMS-G6P and so-called TMS-G1P. Analyses of the Krebs cycle intermediates as their trimethylsilyl derivatives have been carried out on a temperature programme (Horii et al, 1965; Dalgliesh et al, 1966).

In this study it was found that using a temperature programme starting at 100°C and increasing at 2°C or 4°C per minute up to 250°C the TMS derivatives from lactate to G6P could be separated in a single run, taking 65 minutes (at 2°C per minute) or 42 minutes (at 4°C per minute). The analyses at 2°C per minute were used for the radioactive samples on 3% SE-52 or 3% OV-17 on Chromosorb W (AW-DMCS, 80-100 mesh). The analyses at 4°C per minute were on 3% OV-1 on Gas Chrom Q (100-120 mesh). This column packing was superior to all others particularly because of its low column bleed at all temperatures. This resulted in a stable baseline during temperature programming. The column packing 0.125% OV-1 on Corning glass beads used by Sherman et al (1969) whilst giving good separations when operated in the region 200-220°C isothermally, gave rise to very broad peaks when used for temperature programming. The explanation for this lies with the low surface area of glass beads compared with diatomaceous earth and the low level of liquid phase loading possible with the glass beads. This meant that at the starting temperature (100°C) the injected

derivatives would all try to congregate at the top of the column. This would not be possible due to the small amount of liquid phase in this region, it would become saturated and the sample would be spread over a greater volume of the column thus leading to broader peaks. In the most recent work of Sherman et al (1971) 0.1% DC-710 silicone oil on glass was used. Temperature programming with this column packing would be even less likely to succeed. At high temperatures used for isothermal analysis of TMS-sugar phosphates the solubility of the TMS derivative in the liquid phase would be higher than at 100°C, so this combined with the immediate movement along the column by the derivative, would not lead to overloading of the column.

Fumarate and succinate were separated on the SE-52 liquid phase and OV-1, but not on OV-17. The lack of separation on OV-17 was also noted by Horning & Horning (1970). Since OV-17 has a higher proportion of phenyl groups than OV-1 and SE-52 it is hard to understand why this should be. The interaction of the double bond in fumarate with the phenyl groups might have been anticipated to increase the retention time compared with succinate. The separations of the TMS derivatives achieved on OV-17 on Chromosorb W (AW-DMCS) and OV-1 on Gas Chrom Q were superior to those on SE-52 on Chromosorb W (Figures 4.2, p. 118 and 4.3, p. 121). However, SE-52 on Chromosorb W (AW-DMCS) worked very satifactorily for the radioactivity analyses. The radioactivity in each injection recovered from this column was very close to 100%, for a very long period despite increased peak tailing (Figure 4.2a, p.118). This was from a separate batch of 3% SE-52 on Chromosorb W (AW-DMCS) than the one which was investigated during the collection of data (in Chapter 4). Variation of the usefulness

of columns made from separate batches of a particular column packing has been reported by Sherman et al (1971).

Quantitative analysis of carbohydrate intermediate TMS-derivatives

No systematic studies of the quantitative estimation of TMS-derivatives of sugar phosphates by gas chromatography have been reported. Sherman et al (1971) have commented, as in the previous paragraph, about variability of usefulness of column packing for analysis of sugar phosphates. They also mentioned that with a new column several injections of TMS-derivatives were necessary before they appeared out of the column. After sufficient injections the height of the peak obtained reached a plateau, the number of injections varying from column to column. The worst columns did not even show a plateau effect. These observations are identical with those in this study. All the column packings with Chromosorb W (AW-DMCS) showed a pronounced adsorption effect, at least 6-12 injections being necessary before the plateau was reached. For these packings at the end of the analysis, i.e. at 250°C, the next sample to be analysed was injected. This saturated all the sites to which the compounds in this sample might be sensitive. Of course it also provided information on which sensitivity ranges to set the F.I.D. detector and the scintillation ratemeter. It was found necessary to saturate the column at the beginning of each day in this study. These effects were minimal for the 3% OV-1 on Gas Chrom Q column, in fact when the first column which was used was rejected for other quantitative considerations, it was still capable of establishing a plateau within 2 injections. The new column of 3% OV-1 on Gas Chrom Q that was made up was injected with a TMS derivative mixture (see Figure 2.1, p.35) without prior injection of any silylation reagent and with

no noticeable adsorption of any of the compounds present. After several days use two successive injections of the same sample without any prior column deactivation, indeed only 40 minutes after the apparatus being started up, gave essentially identical chromatograms (see Figure 2.2, p.37).

Sherman et al (1971) observed that leaving a column out in the atmosphere caused a profound 'adsorption' effect due to water absorption, and that 'wet' carrier gas could also create problems. Certainly 'wet' carrier gas could well hydrolyse TMS-esters, and for all of the GLC analysis in this study a molecular sieve filter was fitted to the carrier gas stream, and was regenerated at 250°C when a fresh cylinder of carrier gas was necessary.

Allison & Stewart (1971) noted that when analysing samples containing TMS-ascorbic acid, that repeated opening of the sample resulted in a reduction in response. They suspected it was due to moisture being introduced into the sample. Addition of 1 µl of water had a similar effect, as indeed did bubbling the carrier gas through water. The latter effect was abolished by placing a molecular sieve filter between the water and the column.

For the work with SE-52 and OV-17 on Chromosorb W the columns were maintained at 250°C over night with carrier gas still flowing. However, with 3% OV-1 on Gas Chrom Q this was not necessary, and both the heating of the column and the passage of carrier gas overnight were stopped. The column, however, was effectively sealed under nitrogen. The lack of a need to heat the column overnight may have been in part due to the different method of injection used for this column. For the SE-52 and OV-17 on Chromosorb W (AW-DMCS) column packings,

injection of sample was made about 1.5 cms down into the packing, whereas for the OV-1 the sample was injected 1.5 cms above the plug, i.e. into the gas space above the column packing. The top of the column packing was deeper down into the column than usually used so that the injection took place inside the column oven. This prevented injections in the cold spot region. The use of the injection heater although improving the chromatographic separation of TMS-α- and β-G6P brought the useful life of the column rapidly to an end by affecting the compounds with the longest retention times (phosphate esters). Increasing the temperature only made matters worse. It was apparent from this that the top few inches of the column were critical. Direct injection of the sample onto the column resulted in the gradual dissolving away of the liquid phase since the solvent was in the liquid phase after injection. This bared the support to the TMS-compounds. Injection above the top of the column packing allowed the solvent to evaporate before reaching the column packing thus preventing the denudation. In addition non-volatile compounds at worst only reached as far as the glass wool plug. In practice this was what was observed. The injection zones of the SE-52 or OV-17 on Chromosorb W (AW-DMCS) column packings became black very rapidly. As a result the top few inches were replaced every 50 injections. In contrast no colour changes (remained white) were seen for the 3% OV-1 on Gas Chrom Q column packing, the glass wool plug only becoming brown.

Formal investigation of the quantitative responses of the TMS-derivatives during temperature programming were made in this study. There were some fears as to whether it was possible to obtain quantitative data during a temperature programmed analysis. However, with an isolated isothermal detector operating above the temperatures in

the column oven quantitative data was obtained. This problem has been examined in detail by Poulson & Jensen (1971). They examined the molar responses of the n-paraffins from C_6 to C_{32} during temperature programming and found them to increase linearly with increasing number of carbon atoms. In addition they found no difference between injection into the head space above the packing and injection on-column.

Horning et al (1963) have investigated temperature programmed analysis using a F.I.D. and showed that quantitative data can be determined. These results were obtained for methyl esters of fatty acids, and it was also shown that the argon ionisation detector was not suitable for obtaining quantitative data. Ettre et al (1962) also obtained quantitative data during temperature programming and showed that keeping the pressure drop across the column constant, i.e. with decreasing flow rate with increasing temperature, was more satisfactory than attempting to maintain the flow rate by adjusting the pressure drop. This was because the F.I.D. was not so sensitive to flow rate as for instance the katharometer.

Horning et al (1966) have looked at certain urinary acids as their TMS-derivatives and quoted response factors for them. However, no details on the shape of the response curves for each compound were given.

In this study there was a difference in molar response between hexadecane and docosane on the 3% OV-1 on Gas Chrom Q packing (1:1.30) which was close to that expected (ratio 16:22 carbon atoms, i.e. 1:1.375). The relative molar responses of TMS-derivatives were

calculated against TMS-glucose, hexadecane and docosane on this column packing (Tables 4.3, p.124 and 4.4, p.126). The values against TMS-glucose were calculated to compare with results obtained with the SE-52 and OV-17 on Chromosorb W column packings (Table 4.2, p.123). These results were similar considering that they were carried out on a different machine. As seen in Tables 4.3, p.124 and 4.4, p.126 the relative molar response remained the same over the range 0.5 μmole to 0.04 μmole (25 nmoles to 2 nmoles injected onto the column) for the non-phosphate containing TMS-derivatives. The phosphate TMS-derivatives all showed a decreased relative molar response with decreasing concentration. When these results were plotted out (Figure 4.4, p.126) they were found to lie on a straight line for each compound, the phosphate derivatives not passing through the origin. This was verified by measuring the response curve in more detail (Figure 4.5, p.132). The value of C in the equation of the best straight line $y = mx + c$ (Table 4.5, p.133) for the data obtained confirmed what could be seen on the graph that apparently the column packing needed saturating with about one nmole of TMS-phosphate containing derivative before any peak appeared. This was also seen when operating isothermally at 250°C. At the lowest concentrations the response could be seen more satisfactorily when operating isothermally at 250°C because of peak sharpening. The saturation effect was still seen when a sample at the lowest measurable concentration was injected approximately 30-40 seconds after a sample which had high concentrations of phosphate compounds, so that the small sample peak was eluted in the tailing edge of the large sample peak.

Since the response curves for the TMS-derivatives of the acids of the Krebs cycle all passed through the origin, the fact that

the phosphate esters did not do so was because they were phosphate esters, rather than because they were TMS-esters. The TMS derivative of ascorbic acid has been reported to intereact with the column when in low amounts (Allison & Stewart, 1971). However, this could be because the tissue was freeze dried and not purified before silylation. There is of course another possible explanation of this effect, in the chemical reaction itself. The phosphates could bind to the glassware despite it being well silanised. However, the evidence is against this. Sherman et al (1971), Zinbo & Sherman (1970) and Quayle (1959) all reported difficulties with volatile phosphate derivatives adsorbing onto surfaces in the GLC instrument at elevated temperatures, degrading to phosphoric acid which itself caused further degradation. This effect also upset the attempts by Sherman et al (1971) to use the electron capture detector for the estimation of TMS-phosphate esters.

It must be noted in this context the use by Sherman et al (1971) of the thermionic detector for phosphorus compounds. They claimed an enchancement of sensitivity over F.I.D. of one hundred fold. This would be very useful when dealing with tissue extracts where certain phosphate containing compounds were minor since the thermionic detector was also specific for phosphorus. This detector was not used in this study. A very useful arrangement would have been to modify one of the flame ionisation detectors in the heated double detector head on the model 64 Chromatography to be a thermionic detector. By using a splitter of the type describedin Chapter 2 for the radioactive gas chromatography apparatus a constant part of the vapours eluted through the column could be passed to the thermionic detector. The two signals, one from the F.I.D. and the other from the thermionic detector could be fed into two channels of the Rikadenki recorder

(3 channel) so that the two could be readily compared. The unknown factor here is whether the thermionic detector could give quantitative data, and also whether on the columns used in this study the useful range of sensitivity (sub 5 nmoles injected onto the column) was in the region where the amount of adsorption of derivatives was very high. Sherman et al (1971) do not give any indication of whether the hundred fold increase in sensitivity represented a hundred fold decrease in the minimum detectable quantity, or a hundred fold increase in sensitivity over the response given by the F.I.D. It may be that less decomposition occurred on etched glass beads than on the 'inert' diatomaceous earths used in this study.

As the area responses for the TMS-derivatives reported in Table 4.3, p. 124 and 4.4, p. 126 were reproducible and obviously indicated that comparison of area responses could lead to quantitative data, it was of much interest to see whether a constant relationship held between the area of the peak and the peak height. Table 4.7, p. 135 shows this clearly to be so. The greatest variation occurred for the first two compounds to be eluted, TMS-succinate and TMS-fumarate. This was to be expected since they would be most sensitive to variations in the initial starting temperature. There seemed to be two approaches to the problem of cooling the oven down at the end of one analysis before starting another. One was to cool the oven below the initial starting temperature and then to heat up to the starting temperature and allow to stabilise for 5-10 minutes. This was the ideal method. The other method was more practical when working in the routine situation. This was to open the oven door for a set time (7 minutes) - this dropped the oven temperature to 75°C in this study (from 250°C). The door was closed, heating switched on immediately (100°C) and sample injected

1 minute later. It was this method that was used to obtain data in Tables 4.3, 4, 5, 6 and 7, pp. 124,126,133,134+135. It is clearly amenable to automation.

As a rider to this work and in contrast, Hashizume & Sasaki (1967) have shown that tris-trimethylsilyl phosphate could be chromatographed on a polyester column, neopentylglycol succinate on Celite 545, and more importantly the response curve was linear and passed through the origin (range 80-750 nmoles). These authors also state an intention to study biochemically important organic phosphates which has not been realised.

3) Measurement of carbohydrate intermediates in tissue

Whilst organic acids (non-phosphate) from tissue have been studied as their TMS-derivatives (Dalgliesh et al, 1966; M.G. Horning et al, 1967; Horning et al, 1968; Horning & Horning, 1970) no studies have been carried out on the sugar phosphates. The biological applications have been limited to the products of the reaction between ATP and hydrolysed sucrose, catalysed by hexokinase (Eisenberg & Bolden, 1969), and the work with the bovine enzyme D-glucose-6-phosphate-myo-inositol-1-phosphate cyclase (Sherman et al, 1969; Sherman et al, 1971). This again was a purified enzyme preparation with little contamination apart from tris(hydroxymethyl) aminomethane, the buffering ion which was removed by Dowex 50 (H^+) resin.

In this study three approaches to the measurement of carbohydrate intermediates in tissue by gas liquid chromatography were tried (Chapter 5). The simplest, freeze drying the tissue biopsy, of course in no way purified the tissue other than to remove the water present.

The largest amount of sample which could be silylated in 100 µl of reagent (see Figure 5.1, p.138)was 10 mg wet weight (2 mg dry tissue). No attempts were made to see whether quantitative data could be obtained. To help silylation the dry tissue was powdered with a clean spatula. The fine particles however blocked the syringe needle which put a limit on the amount of sample that could be treated. It is unlikely whether the sugar phosphates could be detected in such small amounts of tissue although if the thermionic detector could give quantitative data at the lowest concentration there would be strong justification for investigating the quantitative aspects. For measurement of the radioactivity in individual compounds there would just not be enough in these size samples. Concentration of silylation reagents always led to decomposition of the TMS-ester derivatives, a point noted by Eisenberg & Bolden (1969) for TMS-G6P, so that effectively larger samples could not be applied to the column.

An alternative which has not been tried in this context, but has been used for capillary columns, is to use a small pre-column maintained say at 60°C, which at the beginning of the run would not be attached to the main column. The carrier gas and the solvents from the sample (50-100 µl) would be allowed to vent into the atmosphere and after a set time connected to the main column (using a three-way tap). The small column would be rapidly heated to vaporise the TMS-derivatives, and they would be transferred to the main column. Such small columns would have to be replaced regularly because of effect of the large amount of solvent on them. Thus large samples could be applied to the column without the enormous solvent peak and solvent tailing, and without damaging the main column.

Such a method could equally well apply to the second approach. This involved extraction of the tissue as a method of removing the major contaminants, protein and polysaccharides which interfered with the silylation by demanding much of the silylation reagent present. The ideal extractant should be itself readily removed by simple procedures. 7.2% perchloric acid has been widely used for protein precipitation and intermediate extraction (see for example Opie & Newsholme, 1967) when enzymatic assays were to be used. It was removed itself by precipitation as its insoluble potassium salt, but also precipitated some of the phosphates (Bieleski & Young, 1963). Whilst this was sufficient for enzymatic procedures the amount of perchlorate ion remaining in solution was sufficient to interfere with silylations and to be absorbed irreversibly onto the anion exchange resin AG 1 used in this study. In some early tests during this study it was shown to limit binding of the organic acids to the resin from 25-75%. Another common extractant and protein precipitant is trichloroacetic acid (Bartlett, 1959). However, it also extracted lipids and pigments and required exhaustive extraction with diethyl ether to remove it which would also remove the more non-polar organic acids.

Since extraction and precipitation were not satisfactory enough for the removal of the extractant ready evaporation seemed a much better choice. The lower alcohols were good in this respect. Bieleski and Young (1963) used a complex medium methanol:chloroform: water (12:5:3 by vol) which inhibited phosphatases (in plants) during extraction. Cole & Ross (1966) ground frozen tissue with formic acid and then extracted with 80% aqueous ethanol. Kuksis & Prioreschi (1967) used 75% aqueous ethanol for the extraction of Krebs cycle intermediates from rat liver. In this study 60% aqueous ethanol was

the best solvent tried (Table 5.1, p.142 and Figure 5.2, p.141). Aqueous ethanol had the advantage of precipitating polysaccharide which 7.2% PCA did not. It was also a mild extractant in that it was a neutral solution. To ensure this a few drops of 0.880 ammonium hydroxide were added to the extract before evaporation to dryness. Extraction was carried out at -10°C to prevent any enzymatic degradation of the intermediates present. By grinding to a powder at -80°C and homogenisation in 60% aqueous ethanol at -15°C, rapid mixing of the sample with the solvent was achieved.

The supernatant after centrifugation of the 60% aqueous ethanol extract was dried down over phosphorus pentoxide *in vacuo* and then silylated. By this procedure up to 100 mg of tissue could be silylated in 100 µl of reagent (Figures 5.3, p.145 and 5.4, p.147). As to whether the results obtained by gas chromatographic analysis could be related to give quantitative data, it was shown (Figure 6.1, p.169) that the remaining compounds in the tissue extract did interfere with silylation. However the shape of the standard curve was not affected, and so quantitative data could be obtained by adding a known amount of each of the compounds being studied to an equal volume of the extract. The difference between the two extracts would give rise to the slope of the standard curve in that sample and hence by extrapolation the amount of sample there originally. This is the procedure used for many enzymatic assays to standardise the system. The effect of the tissue extract appeared to be linear with the amount of the extract (Figure 6.2, p.171). The maximum amount of tissue which could be analysed according to Figure 6.2 did not appear to be more than 100 mg. Again this by normal injection methods was not enough to examine the radioactivity in each peak. Interference by other compounds present in large amounts which could be converted to TMS-derivatives on the silylation

of compound being studied has been reported by Margosis (1968). In his work sucrose interferes markedly with the formation of the TMS-ethers of linomyoin.

The third approach was to rid the sample of as much of the impurities as was possible. Since all the compounds which were under study were organic acids and therefore capable of giving anions, ion-exchange purification seemed a useful method of separation. Kuksis & Prioreschi (1967) examined the problem of purifying Krebs cycle acids from rat liver. They concluded that TLC or ion-exchange procedures were the most suitable for the low levels found in animal tissue. TLC was a simple technique and a preliminary separation of the classes of intermediates was possible. However, elution of material from the support was not quantitative. On the other hand, the ion-exchange method was tedious, but elution was quantitative. They passed their extract through a column of Dowex-1 resin (formate form), washing the column thoroughly with water to remove neutral (such as saccharides) and basic compounds (amines). The intermediates were eluted from the resin with 6M formic acid, and the formic acid removed by evaporation under reduced pressure.

Zaura & Metcoff (1969) used Dowex-2 resin (formate form) in a similar fashion, eluting the intermediates with 12M formic acid presumably to keep the elution volume to a minimum. They took care not to leave the extract under vacuum once it had dried because of the volatility of some acids (fumaric and succinic acids).

Bieleski & Young (1963), when purifying the phosphate esters extracted from plant tissue, used Cellex D resin (bicarbonate form) to absorb the phosphate esters. These were eluted with 15% ammonium

bicarbonate solution. The ammonium bicarbonate was removed by lyophilisation. Ralph & Khorana (1961) used triethylammonium bicarbonate as eluant because of its greater volatility. However, Zaura & Metcoff (1969) criticised the use of this method with Krebs cycle intermediates because of losses by evaporation during removal of the eluting agent.

In this study AG 1 resin (formate form) was used, and the intermediates were eluted with 0.5M sodium formate or 2M formic acid. Recoveries were high for all compounds studied (Table 5.3, p. 156).

Prior to this ion-exchange procedure the phosphate in the extract was removed by precipitation as magnesium ammonium phosphate. It should be pointed out that this precipitation should be carried out with care. The pH should not exceed 10 otherwise magnesium hydroxide precipitates causing co-precipitation of sugar phosphates (see Figure 5.5, p. 151). The myxomycete extracts have an advantage that the pigment extracted has a subtle but definite colour change at pH 10 (Sullivan, Jr., 1953). All the water used from there on during the purifications was freshly deionised before use to prevent phosphate contamination.

The main aim of the ion-exchange procedure was to remove glucose. This was a major contaminant since it was present in extracts in large amounts, both of mass and radioactivity. Also the radioactive contamination of glucose was very important since if not removed it would have contributed greater amounts of radioactivity than was present in the tissue carbohydrate intermediates after incubation. The purification using a small column of AG 1 resin prior to incubation was effective (see Table 5.2, p. 155).

The conversion of the sodium formate eluant (sodium formate very usefully prevented elution of the yellow pigment of the myxomycete) to a formic acid solution by passage through a column of AG 50W resin (H^+ form) was inconvenient with regards to time, but it did remove the amino acids. These did not interfere with GLC separation (see Figure 6.4, p.173) since most of them were not converted to TMS-derivatives with BSTFA at the temperature of the reaction (4°C). Normally heating to temperatures in excess of 100°C was required (Stalling et al, 1968).

The aqueous formic acid (0.25M) was evaporated readily at 30°C, and no losses of succinic acid or fumaric acid could be detected using the procedure laid down (Chapter 6) unlike in the studies reported by Zaura & Metcoff (1969). Neither were losses observed during storage of the eluted (33% aqueous ethanol) residue in a vacuum desiccator over phosphorus pentoxide. If the purification procedure was carried out exactingly the dried out residue in the specimen tube was scarcely visible. If the AG 50W resin column was not thoroughly washed with deionised water after regeneration a white solid residue was observed, probably of sodium chloride. Unless the AG 50 column had been recently regenerated by passage of several column volumes of concentrated hydrochloric acid an orange material was eluted from the column.

Interference of the GLC separation by the materials used in the purification did not occur. An aqueous ethanol extract only gave rise to peaks of α- and β-glucose when analysed by GLC after purification (Figure 6.3, p.173). Inorganic phosphate was sufficiently high in distilled water to be a problem, but the use of deionised distilled water overcame this.

Other methods of tissue purification

Solvent extraction of biological material has been used by Dalgliesh et al (1966) using diethyl ether or ethyl acetate extraction of urine (i.e. partition), to examine organic acids. This was a one-step purification before conversion of the acids to volatile forms. Just how effective this was for the more polar members of the Krebs cycle acids (malate and citrate) was investigated in this study (Table 5.4, p.158). Citrate and malate were only poorly extracted whereas succinate and fumarate were readily extracted. M.G. Horning et al, (1967) commented that organic acids containing four or more functional groups could not be extracted by organic solvents. Malate with three functional groups was also unextractable. These results have been confirmed by Horning & Horning (1971).

Other methods which have been used include column chromatography similar to the ion-exchange method used in this study, and TLC. E.C. Horning et al (1967) suggested the use of DEAE-sephadex, an anion exchanger. The organic acids and the sugar phosphates could be absorbed onto the column, the column washed with distilled water and the absorbed compounds eluted with a volatile buffer, pyridine acetate. This was based on the work of Jaakonmaki et al (1967) who examined ethyl-β-glucosiduronic acid in urine. Horning & Horning (1971) now recommend this method for collecting the total acid fraction from tissue extracts. It should be noted that in some separate studies in this laboratory problems were encountered when trying to absorb all the sugar phosphate onto the DEAE-sephadex. Conditions could not be found where 100% of the sugar phosphates were initially absorbed. Also it was difficult afterwards to elute all of the material which had been absorbed.

TLC was used by Kuksis & Prioreschi (1967) as a preliminary separation procedure for their GLC analyses with column packings of teflon as the support. This material gave superior quantitative data compared with Chromosorb W (Kuksis & Vishwakarma, 1963), but gave a very low efficiency column (800 plates per 5 foot column). This low efficiency demanded some degree of primary separation. Using this column packing a linear response curve against amount injected in the region 0.25 to 10 µg of sample was observed. This compares well with the results for the TMS derivatives in this study (0.25 to 5 µg range).

4) Recovery of carbohydrate intermediates from tissue

The recovery of carbohydrate intermediates from tissue by the Method in Chapter 6 was studied by two methods, one with ^{14}C-labelled compounds, and the other by the addition to the tissue of known amounts of mass of each compound. The results are shown in Tables 6.1 and 6.2, pp. 163+165 respectively.

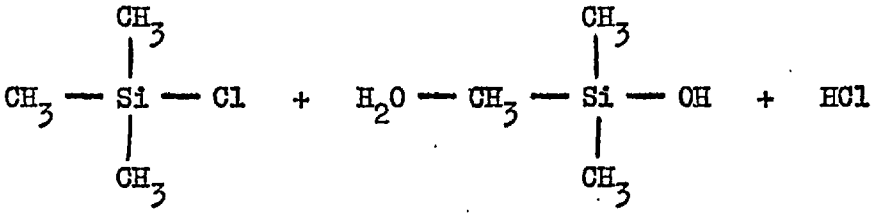
The recoveries of the ^{14}C-labelled compounds were 80% or greater, a quite useful and usable recovery for calculation of quantitative data. These recoveries were in the presence of 1 umole of non-radioactive carrier and about 2.5 g of tissue. The recoveries of small amounts of mass tended to be lower (Table 6.2, p.165) and this was accentuated by smaller amounts of tissue. Kuksis & Prioreschi (1967) observed recoveries in excess of 90% for the members of the Krebs cycle. However, they were using from 10 to 50 mg of each intermediate, i.e. 60 to 300 µmoles, with 5 g of tissue homogenate. They also reported that recoveries remained high down to 1 mg (i.e. 5-6 µmoles), but less than that losses occurred, both overall losses

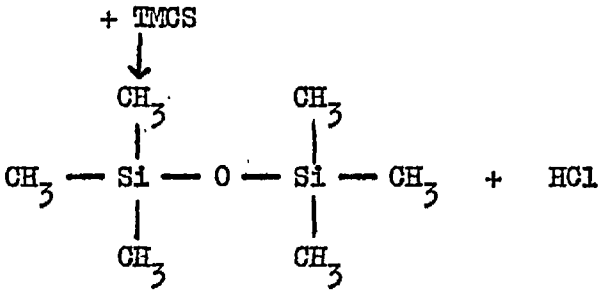
and losses specific to a compound. This seemed to be due to adsorption onto surfaces such as vessel walls. The observation in this study (Table 6.2, p.165) that losses increased with decreased amount of tissue would support this, on the basis that other substances in the tissue extract could fill up the adsorption sites rather than the compounds being extracted.

Thus to ensure good recoveries the amount of tissue purified by this procedure should not be less than 1-2 g. This would mean at the lowest level the heart of an adult rat (about 1 g). This meant that there were no procedures available which could cope with 100 mg to 1 g of tissue unless the normal silylation reagent volume (100 µl) was altered. No advantage would be gained by silylating the aqueous ethanol extract of 200 mg of tissue with 200 µl of silylation reagent compared with 100 mg of tissue and 100 µl of silylation reagent since the concentration of the compounds per µl would be the same. The problems of concentrating the TMS-derivatives reported by Eisenberg & Bolden (1969) for TMS-G6P prevented any movement round this problem. The extraction of TMS-ethers into cyclohexane as used by Margosis (1968) to clean up TMS-linomycin derivatives would not work because of the lability of the TMS-ester derivatives in the presence of added water, and their lability in the absence of excess silylating compounds.

The observation by Sherman et al (1971) that silylation reaction mixtures, if kept long enough, formed two phases neither of which contained the TMS-derivatives was seen in this study, but only if the sample in question had been opened to the atmosphere on several occasions. Such samples when the two phases were observed (this was also seen with the dimethyl ester-trimethylsilyl ethers) did not fume when opened to the atmosphere. Addition of trimethylchlorosilane

(TMCS) restored the single phase system and the peaks for the TMS-derivatives. The two phase system consisted of a colourless upper phase and a yellow lower phase. In the early part of this study a bottle of TMCS which had been left in a refrigerator at 4°C was found to have a broken top and on examination the TMCS was found to have darkened and a yellow oil was found at the bottom. GLC analysis at 100°C of the upper phase revealed many peaks not seen in a fresh bottle of TMCS. TMCS would react with water to give trimethylsilanol which in turn would react with TMCS to give hexamethyldisiloxane.





The action of water on BSTFA would result in the production of mono-trimethylsilyl trifluoroacetamide and trimethylsilanol which again would react to give hexamethyldisiloxane. Since the BSTFA and its breakdown products would be miscible with pyridine it would be the accumulation of hexamethyldisiloxane, a compound with properties like a paraffin, which would form the second phase. The colour in this phase may well be due to the partitioning into this phase of components of the silylation reaction mixture which always gave a yellow colour to such samples.

Radioactive gas liquid chromatography apparatus

The advantage of the liquid scintillation cell described by Popjak et al (1959) over the apparatus with flow-through detectors, the proportional counter (James & Piper, 1961, 1963), the scintillation crystal (Karmen et al, 1962b), a combination of these two (Simpson, 1968), and the ionisation chamber (Mason et al, 1959) was that all of the sample that was passed to the cell was condensed and contained within the measuring system. This meant that a peak containing 100-200 dpm could not only be detected, but providing another radioactive peak was not immediately eluted after it, could also be measured with accuracy. The problem with a flow-through type of detector was that at any one time only part of the peak was inside the detector. By increasing the size of the detector so as to contain all of the peak a very large loss of resolution was observed. Even with a detector of this size a sample would not remain in it for sufficient time to establish accurate data with small amounts of radioactivity. To maintain the resolution achieved on the GLC column the detectors had to be reduced to small volumes such that accurate measurements were difficult with less than 5-10,000 dpm.

Of course an alternative approach to this problem would be to collect the peaks being eluted by trapping them discontinuously in vials containing scintillant, or to which scintillant could be added. This could be carried out manually by an operator each time a peak was observed (either in a radioactivity flow-through counter, or from the mass trace), or could be carried automatically sampling every set time interval. The vials could then be counted in a separate scintillation counter, measuring for as long as was necessary for each sample. Such procedures have been described (Karmen & Tritch, 1960; Karmen et al, 1962a).

However, in this study the intention was to limit the amount of operator control of the analysis. The amounts of radioactivity which were anticipated to be in peaks from samples derived from tissue were low. The flow-through detectors would not have been able to cope despite their qualitative sensitivity. The apparatus described by Simpson (1968) using a proportional counter with a scintillation photomultiplier used in anticoincidence with it, had a very low background (about 1-2 counts per minute) and could detect a bolus of 10 dpm passing through it.

Thus the scintillation cell (Popjak et al, 1959) seemed the most suitable apparatus for this study. The background for the apparatus as described in Chapter 2 (100 cpm for ^{14}Carbon) was higher than could have been achieved. The cell was not cooled below room temperature and the amount of lead shielding was small. The use of complete shielding of the apparatus with an anticoincidence gamma guard counter would have eliminated the high energy background.

This method relied on adequate separation between the peaks of activity, more so than for quantitative measurements of mass, and more so than needed for flow-through detectors. The major disadvantage of the apparatus was when peaks followed each other too closely, and when a small radioactive peak followed a large radioactive peak (James & Piper, 1961). The latter point could be overcome by emptying the scintillation cell once the large peak had been eluted and measured, and adding fresh scintillant to the cell, adjusting the sensitivity range accordingly. Manually this was slow and unpleasant. It was made very much easier by the pneumatically operated automatic scintillant dispenser which was started by a button fixed to the display controls on the gas chromatograph (see Chapter 2 and Figures 2.3 and 2.4, pp. 40 & 42). Automatic range changing (Popjak et al, 1962) was

not used in this work. The presence of an operator for the supervision of the equipment could not be avoided.

Where two peaks were poorly separated (from the radioactive viewpoint), for instance TMS-succinate and TMS-fumarate on SE-52 (Figure 2.8, p. 56), estimates of the radioactivity in each had to be made. This situation could be overcome by the interrupted elution technique. An apparatus for interrupted elution radioactive GLC has been described (Cacace & Perez, 1967). An ionisation chamber was used for the detection of radioactivity (^{3}H-labelled alkanes). The effluent of the GLC column was diverted into the ionisation chamber when a flow ionisation chamber (25 ml) placed between the chamber and the chromatograph detected radioactivity being eluted from the column. When the radioactivity eluted from the column returned to background, chromatography was halted by closing a valve at the end of the column, and also the outlets of the ionisation chamber. The chamber was of sufficient size (2 litres) to contain the whole peak and associated carrier gas and counting gas. Counting was continued long enough to obtain sufficient statistical accuracy. The ionisation chamber was flushed and chromatography continued. The original application was for substances volatile at room temperature, but with a combustion-furnace with copper oxide and zinc dust placed between the column and the flow ionisation chamber, substances of high boiling point were converted to CO_2 and H_2 (Cacace & Perez, 1969).

The problem with this apparatus was that the interruption of the chromatography caused loss of resolution of the peaks still on the column (kinetic diffusion effect). A superior method would be to use the technique of Scott et al (1967) which was originally designed to separate peaks, and then carry out infra-red absorption measurements.

In their method the gas pressure in the column was released from the top when the valve at the end of the column was closed. This resulted in 'backwards chromatography' during the period of interruption of the gas flow. A precolumn was employed so that flow-back past the injection point could occur without loss of the sample. Using this technique peak broadening during the interruption was less than 5% over a period of thirty minutes.

The lack of sufficiently high sensitivity non-specific non-destructive detectors meant that the flame ionisation detector and the argon ionisation detectors were the only possibilities for the measurement of mass. However, the argon ionisation detector did not give a sufficiently linear detector response with increasing concentration. With the F.I.D. a stream splitter attached to the end of the column was used so that a part of the eluted vapours was passed unchanged to the scintillation cell. The constancy of the split ratio with variables such as flow rate and concentration of substance in the eluted vapours (viscosity effect) was investigated by Karmen et al (1963) and Ettre et al (1962). Karmen et al (1963) found that the flow rates down each arm of the splitter were in the same ratio as the ratio of the amounts of mass and radioactivity over a range of flow rates and concentrations. The split ratio of the stream splitter used in this study (1:1.19) would be anticipated to be even less likely to give non-linearity than the 1:8 ratio used by Karmen et al (1963). Their stream splitter was lengths of capillary tube of internal diameter 0.010". They did not observe alterations in the splitting ratio when the arm for radioactive measurements was attached to the collector. The constriction of the stream splitter used in this study was much higher because of the stainless steel wire (diameter 0.0148") inside the capillary

(internal diameter 0.016"). This high degree of constriction and the constant conditions under which the stream splitter was kept (by the heating wire) carefully controlled the split ratio and also the total flow rate during temperature programming (Table 2.1, p. 53).

Some investigations were pursued to see if the combusted gases passing out of the flame ionisation detector could be absorbed into the scintillation fluid thus eliminating the stream splitter. Ethanolamine was added to the scintillant to absorb the ^{14}C-carbon dioxide. Methoxyethanol was also added to give a one phase system. However, this system did not work because of the large amounts (about 3 ml per hour) of water which were formed in the flame ionisation detector condensing into the scintillant.

Bavooshian et al (1971) devised a system whereby the combusted gases from a F.I.D. were passed through a furnace and then to a proportional counter. This arrangement was similar to that used by James & Piper (1961), but had the advantage of greater sensitivity because of F.I.D. detection. It also did not require stream splitting.

Much simpler methods of measuring radioactivity by GLC have been reported. The simplest was that reported by Robbins & Bakke (1967). It involved attaching a pipette which had been wetted on the inside with ethanolamine to the exhaust of a F.I.D. when a peak appeared on the mass trace. The ^{14}C-carbon dioxide was trapped by the ethanolamine and the pipette was washed with scintillant and the washings counted in a vial.

Belham & Neal (1972) condensed the water being emitted from a F.I.D. so as to measure the tritium (^{3}H) radioactivity of a peak. Another simple method was to use a stream splitter and condense the

eluted vapours in a short length of cold column packing, the radioactivity being subsequently eluted. This was used by Karmen & Tritch (1960). However, when dealing with biological extracts the systems which involved continuous monitoring (the flow through detectors and the accumulative detectors) were more satisfactory than the discontinuous detectors since a major radioactive peak might occur without an accompanying mass peak (James & Piper, 1961).

Applications of the Method developed for the study of carbohydrate intermediates in tissue

The applications were carried out using *in vivo* rat heart, an *in vitro* perfused rat heart preparation, and the plasmodia of the myxomycete, *P.polycephalum*. Liver was not used as it gave complex chromatograms (see Figure 4.1, p. 116) and would have made radioactive analyses too complicated for the apparatus used in this study. The interrupted elution apparatus described earlier in the discussion would have been necessary to sort out the peaks. An alternative procedure would be to fractionate the tissue extract. Some preliminary liquid anion exchange chromatography data (K. Blanshard - private communication) has shown that sugars, monophosphates, diphosphates, and Krebs cycle intermediates could be separated as discrete groups. GLC analysis of each of these groups would be relatively straightforward. In this study it was found that 2M formic acid elution of AG 1 resin resulted in a complete separation of G6P, F6P and G1P from FDP.

Yet another approach would be to use higher efficiency columns, such as capillary columns. Since in capillary columns there is no support, the liquid phase being coated onto the inner glass wall, the problem of active sites in the column would be much reduced.

Because of the greater efficiency the elution volumes would be small, this would help the detection. The disadvantage of capillary columns is their low loading capacity (like using glass beads as the support). However, most of the sample to be injected would be solvent so that an arrangement using a small precolumn which would allow the solvent to vent into the atmosphere before passing the derivatives into the capillary column by rapid heating, could be used. This arrangement could have the additional advantage that only the volatile compounds would pass into the capillary column. The non-volatile residues which would be likely to cause loss of column performance on accumulation would not contaminate the capillary column.

The studies (Section 7.1) on the levels of intermediates in the heart of a rat treated with a diabetogenic dose of streptozotocin showed clearly the differences between this rat and a normal rat of the same weight and sex. The values observed for these hearts showed that G6P and citrate were both elevated, and α-glycerophosphate and malate were depressed in the diabetic rat heart. The same trends have been observed in perfused <u>in vitro</u> rat hearts by Newsholme & Randle (1964) and Garland & Randle (1964). The absolute values were not identical, but then the tissue preparations were different, one being <u>in vivo</u> and the other <u>in vitro</u>. Unfortunately no experiments with radioactive glucose were carried out because of the nature of the preparation.

The most interesting results from the experiments which studied the metabolism of [U-^{14}C]-glucose in the <u>in vitro</u> perfused rat heart preparation was the absence of radioactivity associated with TMS-G6P (<u>Figure 7.2</u>, p.187). This occurred in all four metabolic situations

studied, even for the heart perfused with 95% O_2/5% CO_2 when the G6P concentration was greater than 1 μmole/g wet weight of tissue. This result would indicate that the G6P estimated in these experiments originated from the breakdown of glycogen which would be unlabelled at the start of the perfusion, and not from the phosphorylation of glucose. That is not to say that [U-^{14}C]-glucose was not being converted to G6P, this obviously occurred since the intracellular lactate and ∝-glycerophosphate were labelled. An explanation would be that the pool size of G6P originating directly from glucose was very small compared with that from glycogen, and that they were separate pools. Possible evidence for this arose out of further investigations carried out on the extracellular lactate (P. Owen - private communication). This lactate was found to have almost the same specific radioactivity as the substrate glucose as opposed to the intracellular lactate and ∝-glycerophosphate which had specific radioactivities of one-tenth of the substrate glucose (Table 7.2, p. 185).

These observations could all be explained if one considers the physical shape and size of the heart and its cells. If glycolysis (conversion of glucose to lactate or pyruvate via G6P) occurred on the outside of the heart or cell (this would be the method of entry of the glucose) the final product of metabolism, lactate, which freely diffuses through the cell membrane, could diffuse out of the cell without excessive dilution of the specific radioactivity. The other substrate of glycolysis, glycogen, would be inside the cell, and would be separated from the metabolic products of glucose (in fact a concentration gradient of radioactivity would occur falling off towards the centre of the cell). Thus if the glycolysis from glucose proceeded rapidly the concentration of the intermediates would be depressed providing there

were no rate limiting steps in the sequence, and therefore the contribution of [U-^{14}C]-glucose to the whole G6P pool would be very small, possibly as observed unmeasurable.

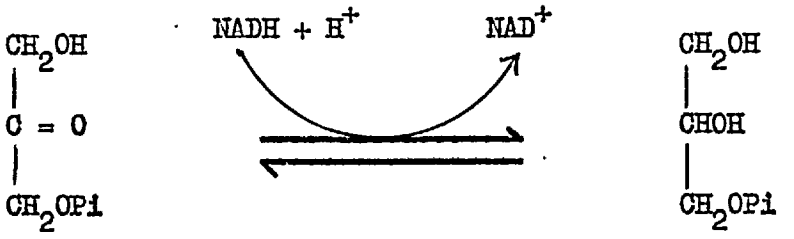
The existence of two pools of G6P in skeletal muscle has been proposed (Shaw & Stadie, 1959). Further investigations were carried out by Landau & Sims (1967), who also looked for a pathway for the synthesis of glycogen which did not have G6P as an intermediate (Antony et al, 1969). However, their results could be equally well interpreted as there being two pools of G6P.

Kalant & Beitner (1971) have shown the probable existence of two metabolic pools for G6P in rat diaphragm, by incubating with ^{14}C-glucose and ^{14}C-pyruvate, and measuring the specific radioactivities of the phosphorylated intermediates. This required 14 diaphragms per result. Measurement of specific radioactivity was by thin layer chromatography after tissue extraction with perchloric acid, and precipitation as barium salts by ethanol. The compounds after being eluted from the thin layer plate were separately assayed for mass (enzymatically) and radioactivity. The specific radioactivities of F6P, FDP and PEP after incubation for 2 hours were very close to that of the substrate, whereas those for G6P and 2PGA and 3PGA (not separated) were only 10-20% of that of the substrate.

Differential metabolism in a multi-type cell system has been observed in the small intestine of the rat. The epithelial cells lining the mucosa were found to be the major producers of lactate (Porteous & Pritchard, 1972a; Iemhoff et al, 1970 and Kimmich, 1970). Since the glucose substrate for these cells was provided by active transport from the mucosal side of the intestine, if this glucose was

radioactively labelled then the lactate produced was hardly diluted with cold material. This lactate then diffused towards the serosal side. However, the carbon dioxide evolved came from all of the cell types and therefore had a variety of precursors, many of them unlabelled, so there was considerable dilution of the radioactivity (Porteous & Pritchard, 1972b).

The effect of anoxia was to increase the specific radioactivities of intracellular lactate and ∝-glycerophosphate. At the same time the intracellular level of ∝-glycerophosphate also rose. The level of intracellular lactate would have risen, but for the fact that it diffused out of the cell. The level of lactate extracellularly rose during anoxia. The rise in ∝-glycerophosphate was because of a need to provide NAD^+ during anoxia.



dihydroxyacetone phosphate ∝-glycerophosphate

Anoxia also affected the amount of radioactivity appearing in the amino acid fraction (Table 7.2, p.185). The extra radioactivity in the amino acids from the anoxic heart which was preperfused in 95% O_2/5% CO_2 was due to the greater amount of glucose taken up (and hence [U-^{14}C]-glucose taken up).

The studies of the incubation of P.polycephalum with [1-^{14}C]-glucose and [6-^{14}C]-glucose showed very clearly the existence of the

pentose phosphate shunt. This pathway was suspected of occurring from observations on the rate of $^{14}CO_2$ production from these substrates, and the rate of ^{14}C-incorporation into lipid (see Appendix 1).

The disadvantage of the isolation procedure for plasmodia was that in order to stop the metabolic reaction quickly enough, the medium could not be separated from the plasmodia. Filtration and centrifugation were not sufficiently rapid. Thus the small amount of radioactivity appearing in lactate could be either extracellular or intracellular, or both. After 1 minute's incubation no activity could be detected in Krebs cycle intermediates or in lactate, but after 6 minutes several of the members of the Krebs cycle, malate, citrate and possibly succinate and fumarate, were labelled in the incubation with [6-^{14}C]-glucose. Very much less activity was associated with these compounds in the incubation with [1-^{14}C]-glucose (Table 7.3, p.191). This was not due to a slower metabolism since G6P and F6P were both labelled from [1-^{14}C]-glucose. The equilibrium values of specific radioactivity were not investigated in this study.

Some studies on the specific radioactivities of carbohydrate intermediates in mouse liver using [^{32}P]-orthophosphate have been reported (Till et al, 1968; Reich et al, 1968). However, 20 livers were necessary to obtain the data, consuming over 1 mCi of [^{32}P]-orthophosphate. The purification procedure was very tedious, a separate method being required for almost every compound. This was evidenced by the number of authors, nine, for this work. In contrast, in this study, one worker could carry out up to six experiments per week. This included growing the organism, incubating with substrate, extraction and purification, radioactive GLC analysis and processing of the data.

Some extensive studies of the specific radioactivities of Krebs cycle intermediates have been carried out by Sauer et al (1970).

Recommendations for the use of this method

A. Type of interest

1. Qualitative studies

Take up to 10 mg wet weight of tissue and freeze dry. Silylate with 100 µl of silylation reagent. It is difficult to detect sugar phosphates in such a sample since these will be very low.

2. Quantitative mass studies

Extract up to 200 mg wet weight of tissue with 60% aqueous ethanol and divide into two equal parts. To one part add 100 nmoles of each compound to be studied. Take to dryness and silylate each part with 100 µl silylation reagent. The difference in responses for each compound in the two parts enables the slope of the standard curve to be calculated. This, like A.1, requires little work up and takes 24-48 hrs.

3. Quantitative mass and radioactivity studies

This requires not less than 1 g wet weight of tissue. Extraction is with 60% aqueous ethanol. The extract is purified as described in Chapter 6. This purification is slow (48 hours). Up to 6 samples can be treated in a working day (9 a.m. to 6 p.m.), using one rotary evaporator. This is the limiting piece of apparatus. The purification on ion-exchange column can be partially automated by using a peristaltic pump (i.e. sucking the columns rather than blowing them). This is easier for the operator.

There is no limit to the number of samples which can be treated per day for methods A.1 and A.2 (at least 40-50). However, not more than 10 samples could be analysed by GLC per day per machine.

B. Compounds to be studied and their concentration

If the interest is in the hexose phosphates isothermal analysis at 230-250°C should be used. This gives maximum sensitivity for the sugar phosphates.

For a complete spectrum of phosphate and non-phosphate organic acids use the programme: 100°C isothermal then temperature programming 100-250°C at 2°C per minute (for radioactivity analyses) or 4°C per minute (for mass analyses). If there is particular interest in lactate, β-hydroxybutyrate or pyruvate, start the programme at 80°C.

For the non-phosphate organic acids there is no lower limit that can be investigated in the silylation reaction mixture, but from tissue less than 40 nmoles would be adversely extracted. For the phosphate organic acids the lower limit that could be studied would be 100 nmole total. If less than 1 nmole is injected onto the GLC column no results at all would be obtained.

C. Choice of Chromatography materials

The 'inert' support, Gas Chrom Q, was superior to Chromosorb W (AW-DMCS). The best liquid phase used was OV-1 although SE-52 and OV-17 were also useful. Non-silicone phases were not suitable for analysis. OV-1 gave only a small bleed during temperature programming and did not need a backing off column.

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APPENDIX 1

BIOCHEMISTRY OF THE MYXOMYCETE, PHYSARUM POLYCEPHALUM

One of the tissues used for recovery data of sugar intermediates in the evaluation of the GLC method was the plasmodia of the myxomycete, Physarum polycephalum. In addition it was used for experiments designed to demonstrate the radioactive-GLC technique. Since little is known about the biochemistry of this organism some studies were carried out so that the data from the radioactive GLC could be better interpreted. In this Appendix a brief outline is made of this organism, its growth and the biochemical studies which have been made by other workers. Then a description is given of some simple experiments which were carried out.

1) Introduction

a) The Myxomycete, Physarum Polycephalum

The true slime moulds, the myxomycetes, are acellular and highly multinucleate in the plasmodial phase, in contradistinction to the cellular slime moulds, the acrasiales. The plasmodium of Physarum polycephalum Schweinitz is a sheet of naked protoplasm which is organised into an extensive network of veins through which the 'sol' protoplasm flows. These veins are prominent and are readily visible to the naked eye. Under the objective of a low power microscope interconnections between the veins can be seen as well as capillaries throughout the 'gel' protoplasm. Within the veins the 'sol' protoplasm streams in one direction with reversals of the direction of flow occurring every one to two minutes. On agar the plasmodium can be many centimetres across.

When nutrients are exhausted, or under some environmental conditions, the plasmodium changes form and becomes a sclerotium. This consists of many thick-walled spherules which are very resistant to adverse conditions. When the conditions become favourable for growth, for example through inoculation into fresh medium, the spherules germinate rapidly and plasmodia appear. Physarum polycephalum has been grown throughout its life cycle in the laboratory although not all stages in pure culture. The plasmodium is formed by the fusion of uninucleate amoebae of differing mating type. In some other myxomycetes the plasmodium can arise within clones of amoebae.

b) Growth of P.Polycephalum in culture

The strain used, except where otherwise stated, was a + i, formed by the mating of two amoeba clones, mating types a and i (Dee, 1962). The plasmodium of P.polycephalum was first grown in pure culture on sterile oat flakes (Cohen, 1939). Pure culture in soluble medium was introduced by Daniel & Rusch (1961). In the present study the plasmodium was grown as described by Carlile (1971), the medium and methods being a modification of those of Daniel & Baldwin (1964).

In liquid culture the plasmodia are tiny (just visible to naked eye) and are known as microplasmodia. They grow vigorously after a short lag phase. During the exponential growth phase the elongated microplasmodia separate into smaller pieces, a large number of dumb-bell shapes being seen. Vigorous streaming occurs along the central axis, being most apparent in the thinner connection between the ends of the dumb-bell. After maximum yield has been attained the microplasmodia present are mostly spherical. These rapidly become microsclerotia, spherules enclosed in a membrane which often breaks open releasing the spherules.

c) Biochemistry of the plasmodium

Kamiya (1968) has carried out extensive studies on the protoplasmic streaming of plasmodia. This aspect has been supported by limited studies on respiration and carbohydrate metabolism. However, almost all this work has been carried out with material grown on oat flakes, the resulting plasmodia probably not being pure cultured. Recently investigations into nucleic acid metabolism and protein synthesis (see Rusch 1970 for review) have been carried out with plasmodia grown in pure culture.

Since pure cultured material is now available it was decided to investigate the carbohydrate metabolism of plasmodia. Already Carlile (personal communication) has investigated with pure cultured material whether the plasmodium can survive anaerobically or not, and supports Kitching & Pirenne (1940) who reported that it could not survive anaerobically. Other workers (Seifriz & Urbach, 1944; Loewy, 1950; Allen & Price, 1950) contended that it could survive.

Respiration and the effect of inhibitors has been studied by several workers (Seifriz & Urbach, 1944; Allen & Price, 1950; Ohta, 1954). The effect of carbon substrates to increase respiration has been reported by Ohta (1954), only glucose, acetate and formate being effective of those studied.

Differences between plasmodia and sclerotia in the content of a glycogen-like polysaccharide has been observed (Sullivan, Jr., 1953). A large decrease in the level of this polysaccharide accompanied the formation of sclerotia from plasmodia induced by either transfer to salts medium (McCormick & Rusch, 1969) or, by the addition of mannitol (to 0.5M) to the growth medium (Chet & Rusch, 1969).

Korn et al (1965) reported the fatty acid composition of the plasmodia when grown on oat flakes. However, lipid droplets and particles of oat can be seen in the veins of plasmodia when grown in this manner and thus the true lipid composition (i.e. less the fat content of the oat flakes) may be different.

2) Methods for the experimental work with myxomycete plasmodia

a) The culture of microplasmodia

The microplasmodia were grown in standard medium; composition as below:-

Glucose	10g
Peptone (bacteriological)	10g
Biotin	5mg
Thiamin hydrochloride	42mg
KH_2PO_4	2000mg
$CaCl_2$ $2H_2O$	900mg
$MgSO_4$ $7H_2O$	600mg
Ethylenediaminetetracetic acid (disodium salt)	224mg
$FeCl_2$ $4H_2O$	60mg
$ZnSO_4$ $7H_2O$	34mg

Distilled water to 1 litre.

The pH was adjusted to 4.6 with concentrated HCl*. The medium was dispensed in 50 ml amounts into 500 ml Erlenmeyer flasks and autoclaved at 15 lbs per square inch for 15 minutes. Haem, an essential growth requirement, was made up separately since it is unstable at pH 4.6. Haemitin (25mg) was dissolved in 1% NaOH (50 ml) and autoclaved at 15 lbs

* for semi-defined medium citric acid (3.54g) was included in the medium, 10% NaOH being used to adjust the pH to 4.6.

per square inch for 15 minutes. It was stored at -5°C. The haem was added to the culture medium just prior to inoculation (0.5 ml per 50 ml).

Inoculations were routinely made from cultures near maximum yield. A 2% inoculum gave maximum yield after 85-96 hours and a 5% inoculum after 72 hours. A weekly routine was established with a 2% inoculum on a Friday and a 5% inoculum on a Tuesday. Inoculation with microsclerotia took a minimum of seven days to give maximum yield. Microsclerotia were formed two days after maximum growth and remained viable for several months.

Plasmodia were also grown on sterile oat flakes (Quaker Quick Porage Oats) in 500 ml Erlenmeyer flasks at 24°C in the dark. Near to maximum yield the plasmodia migrated up the walls of the flask away from the oats. They were then harvested with a sharp knife.

b) <u>The incubation conditions used for experiments with micro-plasmodia</u>

The cultures of the slime mould were centrifuged (1000 *g*, for 2 minutes) and shaken twice with three volumes of the medium to be used in the incubation. Unless otherwise stated this was standard medium lacking glucose and peptone.

Two incubation conditions were used. One procedure was to incubate in conical flasks (50 ml) in a heating bath at 24°C, the flasks being clipped onto a tray which was reciprocally shaken (50 cycles per minute). The incubation suspension was preincubated for ten minutes prior to the addition of substrate. The flasks were covered in aluminium foil to keep out light.

Alternatively incubations were carried out in round-bottom flasks (100 ml) fitted with spargers. The flasks were stood in a water bath (at 24°C) without being shaken. A gas mixture (95% oxygen/ 5% carbon dioxide) was passed through the incubation mixture (0.6 litres per minute). Only standard medium lacking glucose and peptone was used because other media caused foaming. The medium was flushed with the gas before substrate was added.

Radioactive ^{14}C-carbon dioxide produced during incubation was absorbed from the perfusion gas by bubbling it through ethanolamine: ethanol 1:1 v/v (40 ml). Carbon dioxide was not retained in the medium because of the acid pH. The tubes containing ethanolamine:ethanol were used for ten minute periods before being replaced.

c) The extraction of lipid from plasmodia

The centrifuged plasmodia were shaken with at least 5 volumes of chloroform:methanol 2:1 v/v, further methanol being added to keep the mixture as a single phase. The plasmodia were extracted overnight. With samples of large volume (required for fatty acid analyses) the solid was removed by vacuum filtration through chloroform washed filter paper. With smaller samples (for the estimation of total lipid formed during incubation) the solid was centrifuged (3,000 g, for ten minutes). The supernatant was carefully removed and in some experiments the pellet was put aside for glycogen assay. The supernatants were washed with one volume of saline (0.9%).

For the ^{14}C-incorporation experiments, 0.1 ml samples were taken for radioactivity analyses. Larger samples could not be used because under these conditions the myxomycete's yellow pigment caused severe quenching of the scintillation process.

d) The preparation of methyl esters of the Krebs cycle intermediates in tissue

This was a modification of the method of Barnett et al (1968) necessitated by working with myxomycete plasmodia.

The frozen tissue pellet was blended in 6% HCl in methanol (7 volumes per gm tissue) for 30 seconds. The homogenate was centrifuged (2,500 g for 20 minutes) and the supernatant decanted off. The pellet was re-extracted with a further 20 ml 6% HCl in methanol. The two supernatants were combined and anhydrous sodium sulphate added (3 g per g tissue) with vigorous shaking. After allowing to stand for 30 minutes it was filtered through a sintered glass funnel. The filtrate was heated under reflux for two hours. It was then poured into a separating funnel with an equal volume of water. n-Heptane (15 ml) was added and the funnel shaken. The aqueous layer was run off and extracted with 2 x 10 ml chloroform. The combined chloroform extract was washed with saturated sodium carbonate solution (2 ml) and then with 2 x 2 ml distilled water. The final extract was dried with anhydrous sodium sulphate (1 g) for one hour. The chloroform was removed, the solid being washed with chloroform (2.5 ml). The combined extracts were reduced in volume to less than 1 ml under vacuum (water-pump) using a rotary evaporator (at 20°C). The concentrate was transferred to a tapered centrifuge tube and further concentration carried out with a stream of argon to that appropriate for GLC analysis.

e) The preparation of methyl esters from fatty acids in the lipid extract of microplasmodia

The washed chloroform extract was evaporated to dryness under vacuum. Methanol:benzene:sulphuric acid (20:10:1 by vol) (50 ml) was

added to the lipid material and refluxed for 90 minutes. This solution was extracted with 25 ml petroleum ether 40-60°C after previously adding 25 ml distilled water. The lower phase was run off and the upper phase washed three times with distilled water (10 ml). The petroleum ether extract was evaporated to dryness using methanol to remove any water. The dried material was dissolved in petroleum ether (1 ml) and appropriate dilutions made for GLC analysis.

f) The analysis of glycogen from plasmodia

The plasmodia were centrifuged minimising as far as possible the time from sampling to extraction. After decanting the supernatant, 40% KOH (2 ml) was added and the pellet resuspended. The tube was placed in a water bath at 100°C for 30 minutes. Absolute ethanol (8 ml) was then added and the tube shaken. The tube was stored at -20°C for at least two hours, or overnight. It was centrifuged (2000 g for 10 minutes) and the supernatant carefully decanted. The pellet was dissolved in distilled water (2 ml) and absolute ethanol (8 ml) added. The precipitate was centrifuged as before. The pellet was again dissolved in water (2 ml), any insoluble material being removed by centrifugation. The supernatant was carefully removed and added to absolute ethanol (8 ml). The very fine precipitate was centrifuged again. The supernatant was removed. This precipitate was shown to be glycogen-like.

Estimation of the mass of the glycogen and its radioactivity was determined by first hydrolysing it in 2M HCl (4 ml, or 2 ml when low levels of glycogen were expected) at 100°C for two hours. A drop of universal indicator was added and the solution neutralised with 30% KOH. Assays for glucose were carried out on this neutralised solution, diluting as necessary. Glycogen was expressed as the equivalent of

glucose released on hydrolysis. 0.1 ml of the neutralised solution was used for the determination of radioactivity, an extra 0.2 ml distilled water being added to prevent potassium chloride precipitating from the scintillant. The isolation method outlined above removed all the free glucose as judged by the radioactivity of the zero time samples.

g) The manometric assay of the respiration rates of plasmodia

Manometry was carried out using a Braun Warburg Apparatus. Conventional techniques for constant volume analyses were used. Incubations were carried out at 25°C. Standard medium lacking glucose and peptone was the medium used for suspension of plasmodia and was flushed with air prior to incubation.

The total volume of fluid in each flask was 3.2 ml, 3.0 ml in the side arm and main compartment, and in the centre well 0.2 ml. For measurements of oxygen uptake 20% KOH (0.2 ml) was placed in the centre well to remove carbon dioxide, in all other flasks distilled water was used. After equilibration, readings were taken for 30 minutes to examine basal rates (for post-maximal material 60 minutes was necessary). Then the contents of the side arm (if any) were tipped in and measurements taken for up to 60 minutes.

h) Attempts made to measure carbohydrate intermediates from plasmodia using enzymatic methods

The extractants of tissue which were used, 7.2% perchloric acid and 60% aqueous ethanol, both very effectively extracted the yellow pigment. This pigment unfortunately absorbed very strongly at 340 nm, the wavelength used for studying the $NAD^+ \rightarrow NADH$ transitions. This problem could not be overcome by dilution of the sample since it was

then impossible to measure the very small changes due to the presence of the particular carbohydrate intermediate being assayed.

The pigment could be extracted with either sec-butanol or tert-amyl alcohol in acid conditions. In alkaline conditions no extraction occurred. After extraction the aqueous extract had to be evaporated to dryness to remove the alcohol which interfered with all the enzyme assays. This was relatively inconvenient, and the GLC method was studied as an alternative method of measuring the carbohydrate intermediates.

Measurement of citrate in the medium as described in the next section was possible since the level of pigment in the medium was much lower than in the plasmodia, and also the level of citrate being measured was high.

1) Analysis of citrate in the medium

Citrate was assayed by the citrate lyase method (Moellering & Gruber, 1966) using the reactions:

a) citrate $\xrightleftharpoons{\text{citrate lyase}}$ oxaloacetate + acetate

b) oxaloacetate + NADH + H^+ $\xrightleftharpoons{\text{malate dehydrogenase}}$ malate + NAD^+

The neutralised PCA treated medium samples were appropriately diluted and 0.5 ml added to cuvettes containing:

0.05M triethanolamine buffer + 0.2mM $ZnCl_2$ pH 7.6	2.0 ml
NADH (0.125% w/v)	0.1 ml
Distilled water	0.4 ml

5 µl of lactate dehydrogenase and 5 µl malate dehydrogenase were then added to the cuvette and the extinction at 340 nm was recorded until a stable reading was obtained. The change in extinction which

was brought about by the addition of citrate lyase (made up in 10 mM triethanolamine buffer + 0.3 mM $ZnCl_2$ pH 7.6) was used as a measure of the citrate concentration. 20 µl of a 1 mM solution was added to the reaction mixture. A blank run using distilled water instead of sample was carried out, and the results for the unknowns corrected appropriately.

3) Results of experiments carried out with myxomycete plasmodia

a) The growth of plasmodia on standard medium

Standard medium (semi-defined medium with citric acid omitted) was inoculated with material from cultures grown on semi-defined medium (Carlile, 1971). Incubation gave rise to very stringy plasmodia which became entangled to form a large lump. This lump was not broken by the vigorous swirling action of the shaker. Vigorous shaking by hand did not break it up until the culture approached maximum yield. The plasmodia have been since maintained on the standard medium for thirty months (150 subcultures). During this time the clumping phenomenon has largely disappeared, but the microplasmodia are larger than those grown in semi-defined medium.

The dry weight yields of plasmodia grown in semi-defined and standard media are shown in Table A.1, no difference being observed.

The level of citrate in semi-defined medium and standard medium during growth was assayed enzymatically. Failure to utilise citrate was observed during the growth phase (see Table A.2). Twelve months after this experiment, the investigation was repeated (see Table A.3) with different strains of plasmodia. Again no utilisation was observed justifying the omission of citrate from the semi-defined medium.

(contd. on p.259)

Table A.1.

The dry weight of plasmodia grown in semi-defined medium and standard medium

Both groups of three in the first set were inoculated (1%) with material from semi-defined medium. 1% inoculations were made into each of the two groups of three in the second set from the corresponding group in the first set. Similar 1% inoculations were made into the two groups of three in the third set from the corresponding group in the second set. The plasmodia were harvested by centrifugation (1000 g for 5 minutes) washing the pellet twice with distilled water. The pellet was added to a weighed watch glass and heated at 100°C for twenty-four hours and reweighed.

Set Number	Semi-defined medium	Standard medium
1	433 mg) 446 mg) Av. 430 mg 411 mg)	433) 454) Av. 445 mg 452)
2	446) 456) Av. 450 mg 449)	467) 425) Av. 446 mg -)
3	460) 444) Av. 450 mg 446)	460) 465) Av. 460 mg 455)

Table A.2.

The level of citrate in semi-defined medium during growth of plasmodia previously grown in standard medium

Semi-defined medium was inoculated (1%) with material from standard medium (it had been grown through forty subcultures in this medium).

Aliquots (2 ml) of the medium were taken at the times indicated and treated as above.

Time in hours	Concentration of citrate (mM)
0	10.5
24	10.5
48	9.0
72	8.4
96	8.9

Table A.3

The level of citrate in semi-defined medium during growth of different strains of plasmodia

Three flasks of semi-defined medium were inoculated (10%) with plasmodia of each of the strains below. Samples (1 ml) of the media were taken at the times indicated and deproteinised with perchloric acid (7.2%). After neutralisation aliquots were taken for enzymatic analysis.

Strain	2 hours	24 hours	48 hours	72 hours	96 hours	144 hours
a + i	14.0*	14.0	14.0	14.0	12.0	12.0
i + A7015 [1]	14.5	15.5	15.0	13.0	14.0	13.5
Poulter 1. [2]	16.0	16.5	13.5	15.5	13.5	14.0

* concentration as mM

1 i + A7015 - made by mating amoebae i and A7015 (Poulter & Dee, 1968).

2 Strain provided by Dr. Russell Poulter, Dept. of Genetics, University of Leicester.

b) The utilisation of glucose in media by plasmodia

The uptake of glucose was measured for plasmodia which had been centrifuged and resuspended in the same supernatant, and for plasmodia which had been centrifuged and added to fresh standard medium. These values were compared with the rate observed before centrifugation (see Table A.4).

Since the rate was not adversely affected, centrifuging and washing the plasmodia to remove unwanted materials prior to incubation was carried out routinely.

When washed plasmodia were resuspended in glucose-free standard medium, added glucose was utilised at the normal rate. Microscopic observations showed that 24 hours after all the external glucose had disappeared there was no formation of sclerotia, and that streaming still occurred. In similar observations (M.J. Carlile - personal communication) no sclerotia had been observed in glucose-free semi-defined medium after 14 days incubation. Transfer of active plasmodia into standard medium lacking both glucose and peptone resulted in complete solerotium formation within 24 hours.

An experiment was carried out to determine whether the presence of peptone was responsible for the prevention of formation of sclerotia (see Table A.5).

The ability of the plasmodia to survive as plasmodia in glucose-free standard medium may be due to utilisation of internal glucose storage compounds or, utilisation of peptone, or both. A test for glycogen (see Section A.2.f) was carried out with a culture near maximum yield. The glucose equivalent of glycogen represented 15% of the dry weight.

(contd. on p. 262)

Table A.4

The effect of centrifugation and fresh medium on the uptake of glucose by plasmodia

A 100 ml culture near the end of the phase of rapid growth was divided into three. Two of the three parts were centrifuged (1000 g for 2 minutes), one merely being resuspended and the other, after removal of the supernatant, being added to fresh standard medium. The three parts were replaced on the shaker and samples (1 ml) taken over eight hours. From measurements of the glucose levels by glucose oxidase, and determination of dry weight, the rate of uptake was calculated. Corrections were made for the reduction in dry weight by the samples removed.

Conditions	Rate of uptake mg glucose/ g dry weight/hour
original flask	28.3
centrifuged - resuspended in same supernatant	34.0
centrifuged - added to fresh medium	30.6

Table A.5

The effect of glucose and peptone on the formation of solerotia from plasmodia

100 ml standard medium culture close to maximum yield was divided into four and each centrifuged (1000 g for 2 minutes). Each pellet was taken up in 25 ml of the above media and incubated at 24°C in the dark.

Time (hours)	Modifications of standard medium			
	Glucose 1% Peptone 0%	Gluoose 0% Peptone 0%	Glucose 0.016% Peptone 1%	Glucose 0% Peptone 1%
3	Very irregular shapes.	Mainly spherical plasmodia active streaming	Very irregular shapes mainly stringy.	Stringy lengths, some dividing into spherical shapes.
6	Spherical plasmodia.	Mainly spherical plasmodia some streaming in long pieces.	Very irregular shapes. Streaming.	Very irregular shapes. One sclerotium seen.
24	Many solerotia (40%) but some streaming seen.	Almost all solerotia.	Two sclerotia seen but otherwise plasmodia with active streaming.	One sclerotium seen, active streaming.
48	Mainly sclerotial (>70%) some spherioal plasmodia seen.	All sclerotia.	Some sclerotia (5%) but still many plasmodia with active streaming in others.	Active streaming some sclerotia (5%).

c) Investigations on the glycogen-like material isolated from plasmodia

The material isolated by the procedure mentioned in section A.2.f. was examined. Dilute (0.025M) iodine solution was added to solid on a watch glass. When viewed with a microscope the characteristic red-brown colour obtained with glycogen could be seen. A spectrophotometric assay was also carried out. When iodine in potassium iodide solution (0.025M) was added to a solution (5 mg in 3 ml) of the polysaccharide the colour kept fading before it became permanent (oyster glycogen did not show this effect). Iodine was added to the blank so that the negative peak at 430-450nm was balanced out. The scan was then made (see Figure A.1). There was a well-defined peak at 500nm, whereas with oyster glycogen the peak was at 490nm.

Incubations with α-amylase gave the same amount of reducing power as the same amount of oyster glycogen. Further investigation by β-amylolysis indicated it was similar to rabbit liver glycogen. The value obtained was 45%, the expected range for glycogen of different origins is 40-55% (this analysis was kindly performed by Dr. B. J. Ryman).

The ease of solubility in water was another glycogen characteristic, and so was the ready precipitation with low percentages of ethanol to give a very fine precipitate. As a result of these experiments, and of the results reported by Goodman & Rusch (1969) this material was called glycogen and treated as such during further experiments.

Samples were taken of plasmodial material during the experiment recorded in Table A.5. The glycogen was isolated and analysed (see Table A.6).

(contd. on p.266)

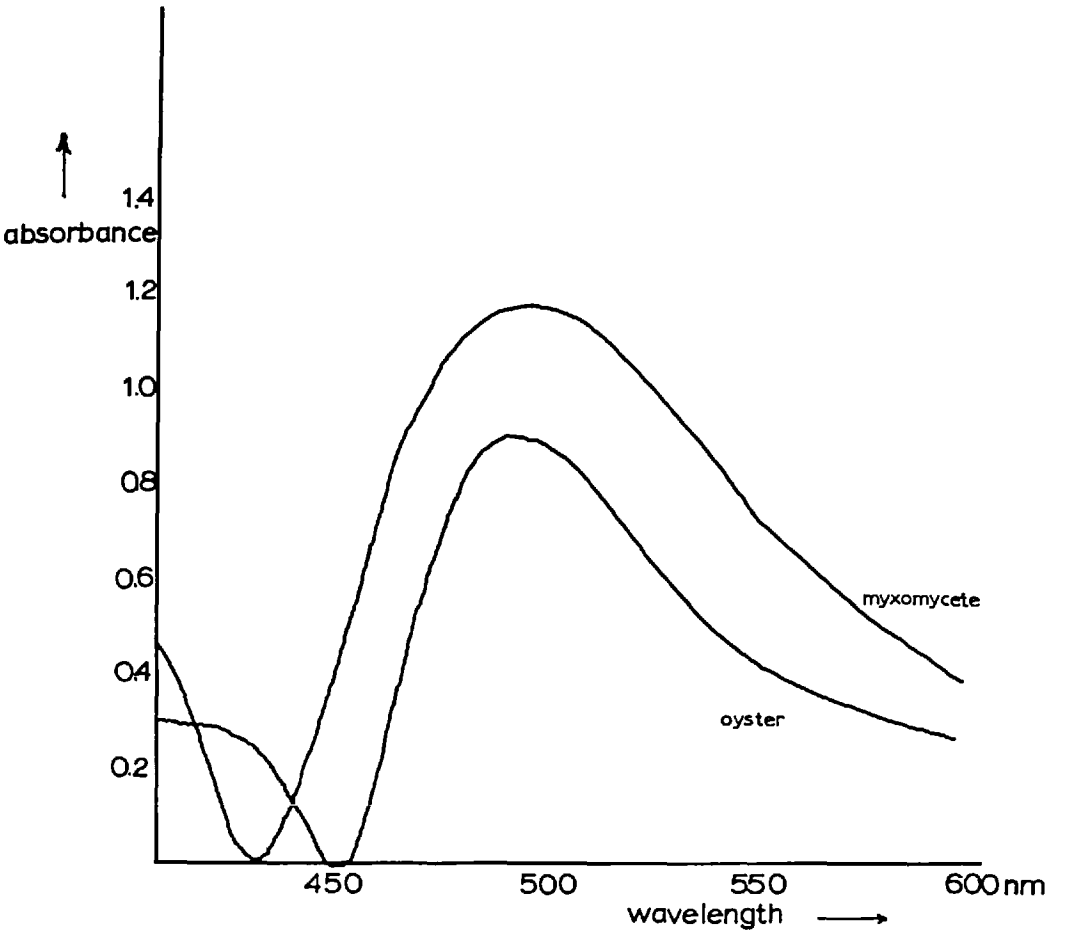

absorbance
1.4
1.2
1.0
0.8
0.6
0.4
0.2
myxomycete
oyster
450
500
550
600nm
wavelength

Figure A.1 The absorption spectrum of the iodine-polysaccharide complex formed between iodine and the plasmodial polysaccharide and oyster glycogen.

5 mg of the plasmodial polysaccharide isolated by the "glycogen" procedure and 5 mg of oyster glycogen were each dissolved in 2 ml water. 0.025M iodine in potassium iodide was added to generate the reddish-brown colour of the complex. This reagent was added to the blank cell until the minimum absorbance in the region 430-450 nm was zero.

Table A.6

The glycogen levels during the formation of sclerotia in different media

100 ml standard medium culture close to maximum yield was divided into four and each centrifuged (1000 g for 2 minutes). Each pellet was taken up in 25 ml of the above media. Aliquots (1 ml) of the incubation mixture were removed at the times indicated. Glycogen was isolated as described in Section A.2.f. and determined as its glucose equivalent after hydrolysis.

Modifications of the standard medium				
Time (hours)	Glucose 1% Peptone 0%	Glucose 0% Peptone 0%	Glucose 0.016% Peptone 1%	Glucose 0% Peptone 1%
0	149*	-	157	168
2	149	123	156	124
4	133	88	103	99
22	146[1]	45[2]	13[3]	14[3]

* µg glucose released after hydrolysis of glycogen isolated from 1 ml incubation mixture.

1 mainly sclerotia (70%)
2 completely sclerotia (100%)
3 mainly plasmodia, few sclerotia (5%)

See Table A.5

Synthesis of glycogen was demonstrated by incubation of plasmodia in low glucose (5.5mM) standard medium. A control flask without added glucose showed a reduction of the glycogen level (see Table A.7).

The use of [U-^{14}C]-glucose indicated that a large part of the glucose taken up by the plasmodial suspension was converted to glycogen. It was then felt necessary to demonstrate net synthesis of glycogen unambiguously. Because of the high levels of glycogen in the plasmodia normally used, plasmodia were incubated in glucose-free standard medium for 20 hours. No sclerotia could be seen after this preliminary incubation, but the level of glycogen was reduced. Incubation with [1-^{14}C]-glucose and [6-^{14}C]-glucose was carried out (see Table A.8) and showed parallel uptake of ^{14}C-glucose and increase in net glycogen levels.

Further experiments were carried out to study this incorporation over a long period (see Figure A.2, and Table A.9, and Figure A.3 and Table A.10). Large uptake of glucose was observed and more particular large net synthesis of glycogen. The glycogen level began falling as soon as the external glucose was used up (Figure A.3).

d) Oxygen uptake and carbon dioxide production by plasmodia

The rate of oxygen uptake and carbon dioxide production of plasmodia was measured with a Warburg manometer (see Table A.11). The rates were not affected by addition of glucose, pyruvate or citrate when plasmodial material from late in the phase of rapid growth was investigated. The glucose was however taken up by the plasmodia at a rate of 32 mg/g dry weight/hour. Adenosine monophosphate and adenosine

(contd. on p. 276)

Table A.7

The glycogen levels in plasmodia incubated in the absence and in the presence of glucose

A 50 ml standard medium culture near to maximum yield was divided in two and centrifuged (1000 g for 2 minutes). The pellets were taken up in 25 ml glucose-free standard medium. Glucose (25 mg) was added to one flask, both being incubated at 24°C in the dark. Samples (1 ml) were taken at the times indicated. Glycogen was isolated and assayed as described earlier (Section A.2.f.).

Time (Hours)	Low glucose (5.5 mM) standard medium	Glucose-free standard medium
0	1.3 mg *	1.4 mg
0.5	1.3	1.3
1.0	1.4	1.4
1.5	1.5	1.2
2	1.6	1.3
3	1.6	1.1
4	1.8	1.1
5	1.8	1.0
48	not detectable	not detectable

* Glucose released by the hydrolysis of glycogen from 1 ml of the incubation mixture. The concentration of the glucose in the medium was also determined for each sample. The glucose level in the medium was zero after four hours. The rate of uptake of glucose was 31 mg/g dry weight/hour.

Table A.8

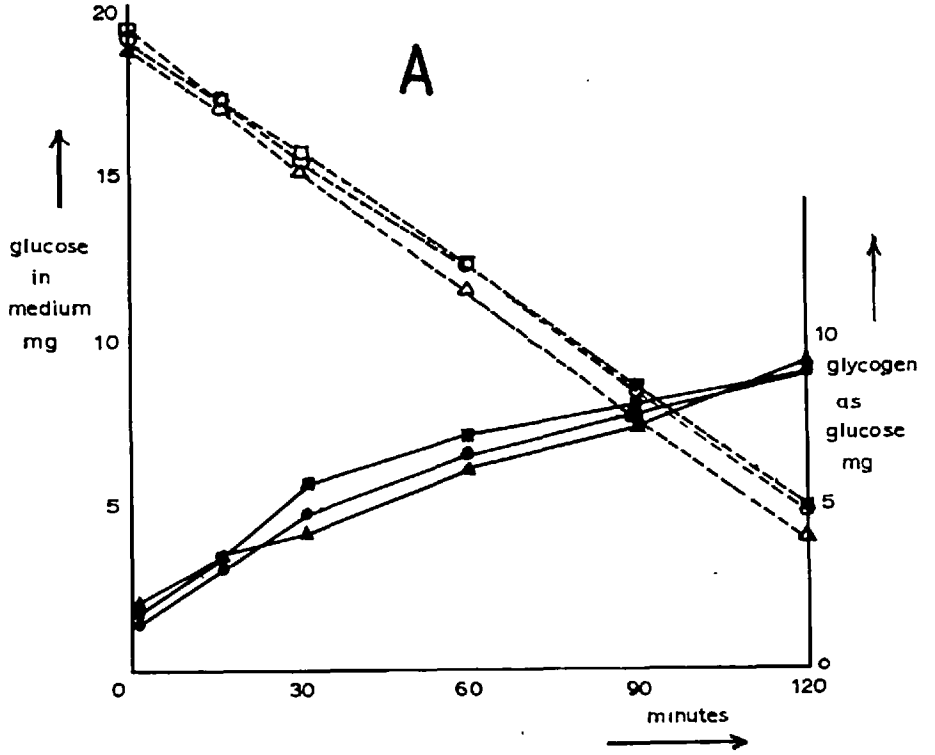
The conversion of glucose to glycogen by starved plasmodia

A culture (100 ml) grown on standard medium was centrifuged (1000 g for 2 minutes). The pellet was added to 50 ml glucose-free standard medium and incubated at 24°C in the dark for 20 hours. This culture was then centrifuged and washed with standard medium lacking both glucose and peptone. It was finally resuspended in 50 ml of this medium. Two (25 ml) aliquots were put into 2 x 100 ml round bottom flasks and perfused with a gas mixture (oxygen 95%/carbon dioxide 5%) as described in Section A.2.b. at 25°C. To one flask 20 mg glucose labelled with [1-^{14}C]-glucose (29 µc/m mole) was added and to the other flask 20 mg glucose labelled with [6-^{14}C]-glucose (33 µc/m mole) was added. Samples (2 ml) of the incubation mixture were taken at one minute, 11 minutes, 21 minutes and 31 minutes. The glucose in the medium was assayed with glucose oxidase. Glycogen was isolated as mentioned before and hydrolysed to glucose. The radioactivity in the medium and in glycogen was measured by taking aliquots of the medium and of the hydrolysed glycogen.

The figures in the table are the differences between data obtained after one minute and 31 minutes. Glycogen increased from 1.5 mg to 4.9 mg equivalent of glucose during the incubation.

	Expt. 1	Expt. 2
Glucose consumed	3.3 mg	3.6 mg
Glycogen produced	3.7 mg	3.2 mg
^{14}C-glucose consumed	0.53 *	0.77 *
^{14}C-incorporated into glycogen	0.46 *	0.52 *

* radioactivity as µc


A
glucose in medium mg
glycogen as glucose mg
minutes
0
30
60
90
120
5
10
15
20

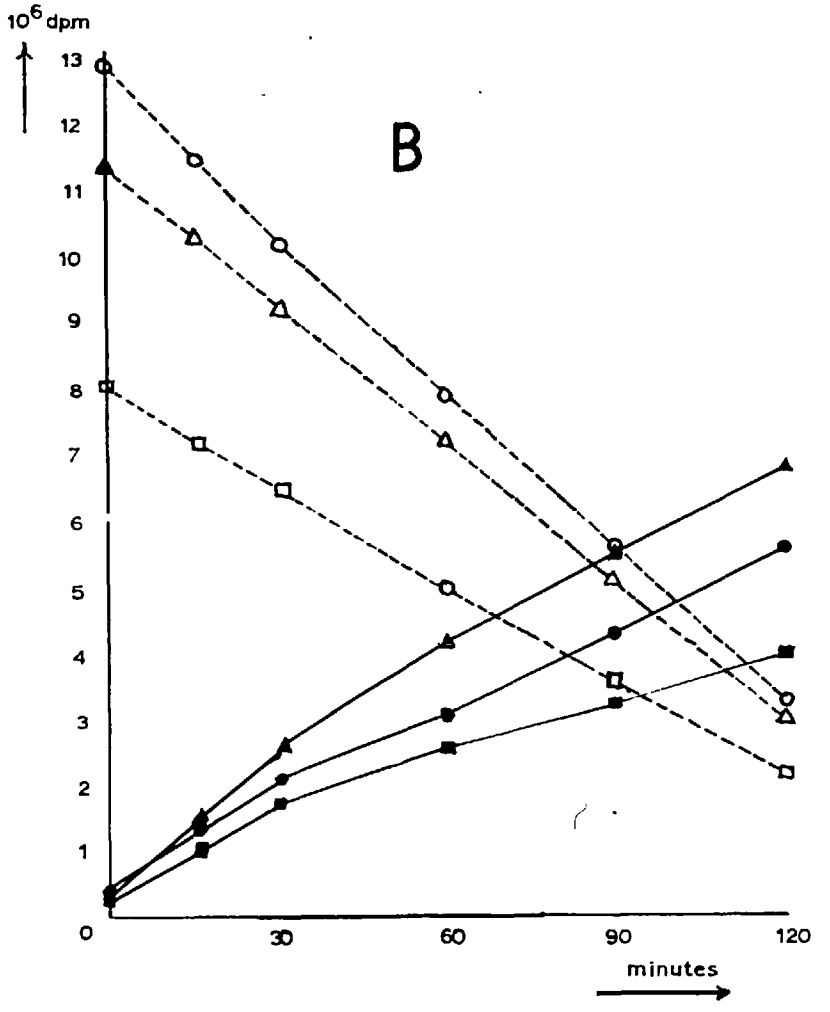

B
10^6 dpm
minutes
0
30
60
90
120
1
2
3
4
5
6
7
8
9
10
11
12
13

Figure A.2 The conversion of glucose into glycogen by starved plasmodia

A. The experimental conditions are those described in the legend to Table A.9.

..... glucose in the medium.

——— glycogen in the plasmodia.

B. The experimental conditions are those described in the legend to Table A.9.

..... ^{14}C-activity in the medium.

——— ^{14}C-activity in glycogen in the plasmodia.

▩ □ [1-^{14}C]-glucose

◉ ○ [2-^{14}C]-glucose

▲ △ [6-^{14}C]-glucose

Table A.9

The conversion of glucose to glycogen by starved plasmodia

A 100 ml culture grown on standard medium was preincubated in glucose-free standard medium for 20 hours. It was resuspended in 100 ml standard medium lacking both glucose and peptone, and three aliquots (30 ml) added to three 100 ml round bottom flasks and perfused with a gas mixture (95% oxygen/5% carbon dioxide) as described earlier (Section A.2.f.) at 25°C.

To one flask 20 mg glucose labelled with [1-^{14}C]-glucose (33 μc/m mole) was added, and to the other flask 20 mg glucose labelled with [2-^{14}C]-glucose (52 μc/m mole). To a third flask 20 mg glucose labelled with [6-^{14}C]-glucose (47 μc/m mole) was added. Samples of medium (2 ml) were taken after one minute, 16 minutes, 31 minutes, 60 minutes, 90 minutes and 120 minutes. Glucose in the medium and plasmodial glycogen were assayed as described in Table A.8 and in Methods.

The figures in the table are the differences between data obtained after one minute and two hours. Glycogen increased from 1.8 mg to 9.0 mg equivalent of glucose during the incubation.

	Experiment 1	Experiment 2	Experiment 3
Glucose used	14.5 mg	14.5 mg	14.9 mg
Glycogen formed	7.1 mg	7.5 mg	7.4 mg
% conversion	49%	52%	50%
^{14}C-glucose used	2.69 *	4.32 *	3.87 *
^{14}C-activity in glycogen	1.77 *	3.08 *	2.50 *
% conversion	66%	71%	65%

* radioactivity as μc_i

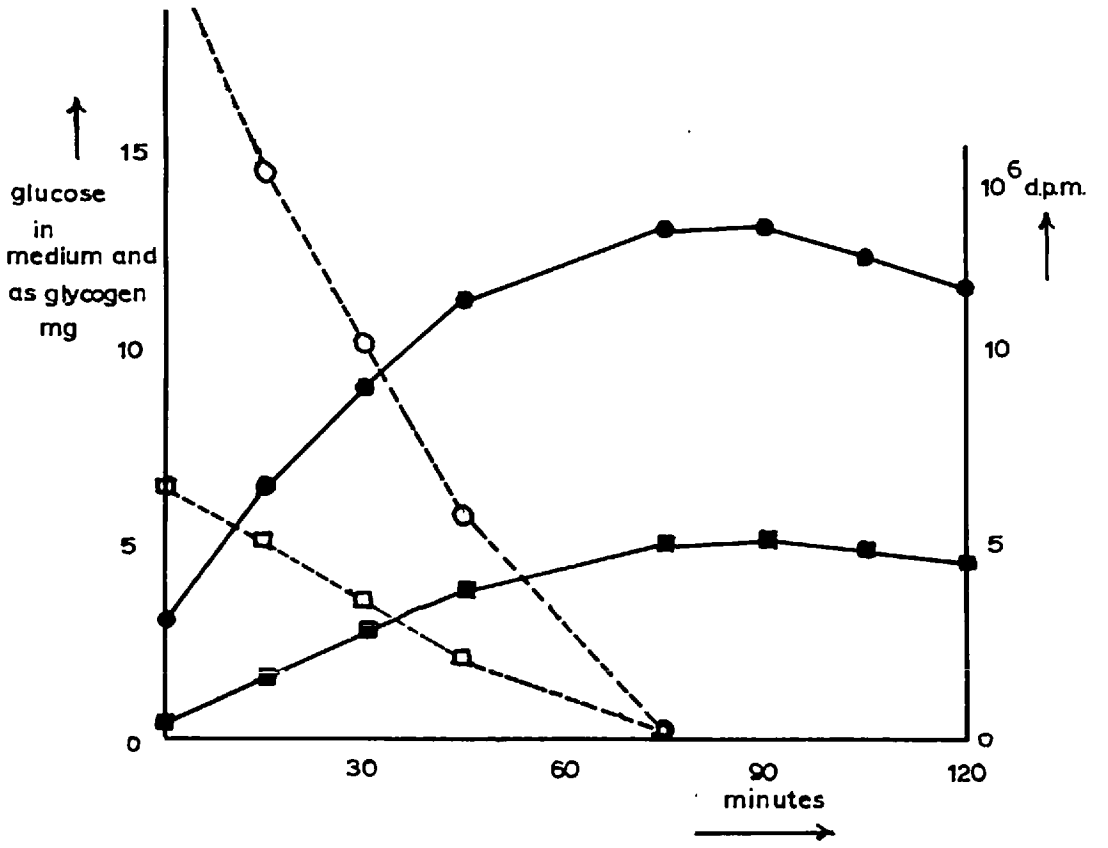
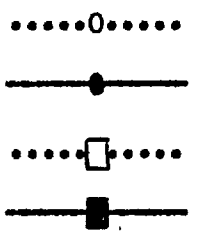

glucose in medium and as glycogen mg
15
10
5
0
10^6 d.p.m.
10
5
0
30
60
90
120
minutes

Figure A.3 The incorporation of [U-^{14}C]-glucose into glycogen in starved plasmodia

The experimental conditions were as described in the legend to Table A.10.



mass of glucose in the medium.

mass of glycogen (as glucose) in plasmodia.

^{14}C-activity in the medium.

^{14}C-activity in glycogen in plasmodia.

Table A.10

The time course of conversion of glucose to glycogen by starved plasmodia

A 50 ml culture grown in standard medium was preincubated, as described in Table A.8, in glucose-free standard medium for 20 hours. It was resuspended in 50 ml standard medium lacking both glucose and peptone. 45 ml of this suspension was added to a 100 ml round bottom flask and perfused with a gas mixture (95% oxygen/5% carbon dioxide) as described earlier (Section A.2.b.) at 25°C.

20 mg glucose labelled with [U-^{14}C]-glucose (30 µc/m mole) was added. Samples of medium (2 ml) were taken at 0, 15, 30, 45, 75, 90, 105 and 120 minutes. Glucose in the medium and plasmodial glycogen were assayed as described in Table A.8 and Methods (see also Figure A.2) except that the plasmodial pellet was extracted with chloroform-methanol (for lipids) before hydrolysis with 40% KOH.

Glucose disappeared completely in 60 minutes. Glycogen increased from 3.0 mg to 12.0 mg equivalent of glucose during the incubation.

Period (Minutes)	% mass conversion §	% ^{14}C-activity conversion *
0-15	60	100
0-30	58	84
0-45	56	80
0-60	49	74

§ % mass conversion = $\frac{\text{increase in glycogen (as glucose)}}{\text{decrease of glucose in medium}} \times 100$

% ^{14}C-activity conversion = $\frac{\text{increase in } ^{14}\text{C-activity in glycogen from plasmodia}}{\text{decrease in } ^{14}\text{C-activity in medium}} \times 100$

Table A.11

The oxygen uptake and carbon dioxide production by plasmodia

Approximately 50 mg dry weight of plasmodial material was added to each flask. Incubation was carried out in standard medium lacking both glucose and peptone at 25°C.

Source of plasmodia	$Q_{O_2}^{air}$	$Q_{CO_2}^{air}$
End of rapid growth phase	14.4 ± 0.5 (11)	12.8 ± 0.5 (8)
1 day after maximum yield	10.3	9.2
2 days after maximum yield	7.1	5.7

The standard deviation is given after the mean. The number of values obtained is given in parentheses.

$Q_{O_2}^{air}$ = µl oxygen/mg dry weight/hour.

$Q_{CO_2}^{air}$ = µl carbon dioxide/mg dry weight/hour.

diphosphate were added to attempt to create apparent anoxia and hence quicker glycolysis, but they had no effect on the $Q_{O_2}^{air}$ and $Q_{CO_2}^{air}$ of plasmodia in the presence of glucose (4 mM).

e) Production of ^{14}C-Carbon dioxide from ^{14}C-glucose

The radioactivity of the carbon dioxide evolved from plasmodial suspensions perfused with 95% oxygen/5% carbon dioxide was measured using [1-^{14}C]-glucose and [6-^{14}C]-glucose. Wide variations in the specific yields were obtained. The results and experimental conditions are recorded in Table A.12.

In each experiment ^{14}C-carbon dioxide was evolved from [1-^{14}C]-glucose at a greater rate than that from [6-^{14}C]-glucose. Figure A.4A shows rates of ^{14}C-carbon dioxide evolved by plasmodia from the active growth phase. Figure A.4B shows the rates of ^{14}C-carbon dioxide evolved by 1 day post-maximum plasmodia indicating a reduction in the preferential conversion to $^{14}CO_2$ from [1-^{14}C]-glucose to [6-^{14}C]-glucose.

The ^{14}C-activity appearing in the lipid fraction extracted with chloroform:methanol after incubation with [6-^{14}C]-glucose was higher than that from [1-^{14}C]-glucose (see Table A.13). The increase in incorporation was shown to continue when there was no more external ^{14}C-glucose (see Figure A.5).

f) The Krebs cycle acids of plasmodia determined as their methyl esters by gas chromatography

The methyl esters of the Krebs cycle acids in plasmodia were prepared (see Section A.2.d.). The plasmodia were grown in semi-defined medium in a 5 litre fermentor flask and were not washed after centrifugation (i.e. some exogenous citric acid present).

(contd. on p.283)

Table A.12

The specific yields of ^{14}C-carbon dioxide evolved by plasmodia from [1-^{14}C]-glucose and [6-^{14}C]-glucose

Plasmodia were suspended, after centrifuging and washing, in standard medium lacking glucose and peptone. Glucose was added to give a 2 mM concentration and was labelled with ^{14}C-carbon (0.1 - 0.5 μc/μmole). A 95% oxygen/5% carbon dioxide gas mixture was perfused through the suspension which was maintained at 25°C, and then passed through 40 ml ethanolamine:ethanol (1:1, v/v). The ethanolamine:ethanol was replaced every ten minutes.

Source of plasmodia	Specific yield C-1	Specific yield C-6	% glucose taken up	Length of incubation
End of phase of rapid growth	0.10	0.03	38	20 minutes
	0.14	0.09	13	20 "
	0.19	0.08	45	20 "
	0.39	0.25	75	45 "
	0.25	0.01	50	10 "
	0.15	0.06	80	30 "
1 day post maximum yield	0.21	0.11	75	20 "
	0.13	0.10	80	40 "

$$\text{Specific yield} = \frac{^{14}C\text{-carbon dioxide evolved during incubation}}{^{14}C\text{-glucose used during incubation}}$$

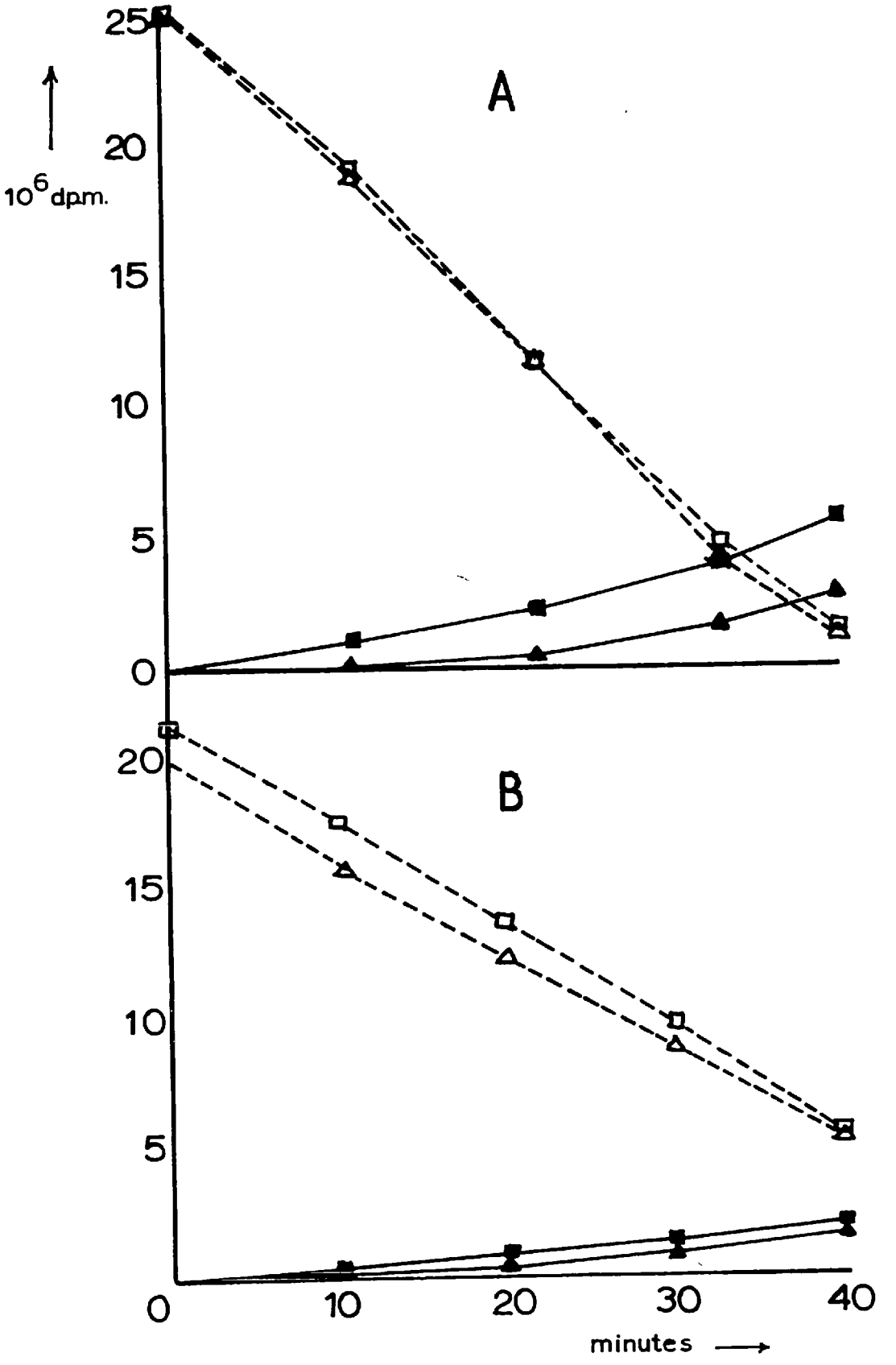

A
B
25
20
15
10
5
0
20
15
10
5
0
10
20
30
40
10^6 d.p.m.
minutes

Figure A.4 The conversion of [1-^{14}C]-glucose and [6-^{14}C]-glucose to $^{14}CO_2$ by plasmodia

The experimental conditions were those described in the legend to Table A.12.

Fig.A was obtained with plasmodia from the active phase of growth.

Fig.B was obtained with plasmodia which were 24 hours post maximum yield.

—□— [1-^{14}C]-glucose in medium —■— $^{14}CO_2$ evolved from [1-^{14}C]-glucose.

—Δ— [6-^{14}C]-glucose in medium —▲— $^{14}CO_2$ evolved from [6-^{14}C]-glucose.

Table A.13

The incorporation of ^{14}C-activity from ^{14}C-glucose into the lipid fraction of plasmodia

Plasmodia from late in the phase of rapid growth were incubated in standard medium lacking glucose and peptone to which glucose had been added (2 mM) labelled with ^{14}Carbon (0.05 μc/μmole), and were perfused with 95% oxygen/5% carbon dioxide for 30 minutes at 25°C. Lipid material was extracted with chloroform:methanol and washed with saline (Section A.2.c.).

In Experiment 3, plasmodial material which had been pre-incubated in glucose-free standard medium for 20 hours was used.

Experiment No.	[1-^{14}C]-glucose		[6-^{14}C]-glucose	
	^{14}C-in lipid	^{14}C-disappeared from medium	^{14}C-in lipid	^{14}C-disappeared from medium
1	0.18 *	0.94	0.27	0.90
2	0.43	2.90	0.75	3.25
3	0.15	2.88	0.28	2.79

* radioactivity units as μc_i

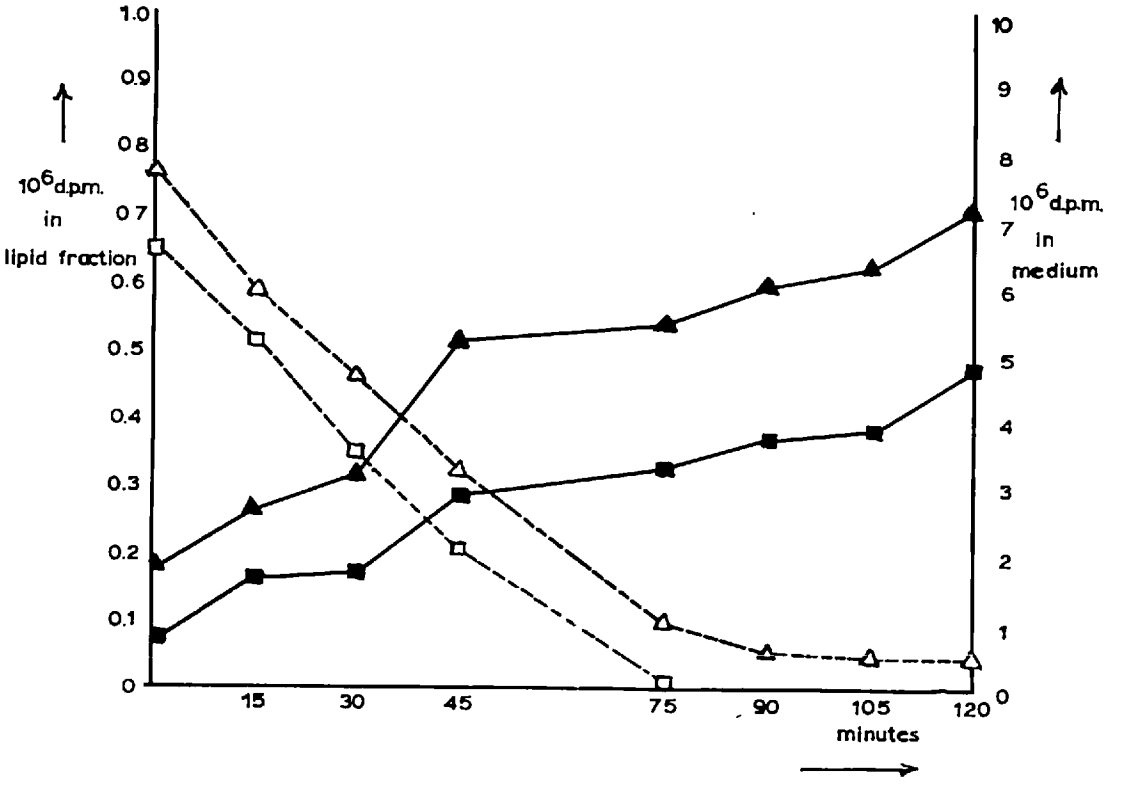

1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
10
9
8
7
6
5
4
3
2
1
0
10^6d.p.m. in lipid fraction
10^6d.p.m. in medium
15
30
45
75
90
105
120
minutes

Figure A.5 The incorporation of ^{14}C-activity into the lipids of plasmodia incubated with [1-^{14}C]-glucose and [6-^{14}C]-glucose

The experimental details were those described in the legend to Table A.13.

—□— [1-^{14}C]-glucose in the medium.

—■— ^{14}C-activity in the lipids after incubation with [1-^{14}C]-glucose.

—△— [6-^{14}C]-glucose in the medium.

—▲— ^{14}C-activity in the lipids after incubation with [6-^{14}C]-glucose.

When aliquots were chromatographed on 10% PEGA on Celite numerous peaks were observed, citric acid being the major peak (see Figure A.6A). Peaks were identified for succinate, fumarate, malate and lactate. The level of lactate was high considering the known volatility of methyl lactate. The presence of these intermediates was indicative of the Krebs cycle operating in plasmodia. The separation of the methyl esters of succinate, fumarate, malate and citrate prepared in the laboratory is shown in Figure A.6B.

g) The fatty acids of the lipids of plasmodia

The fatty acid composition of the strain used in this study grown in standard medium was quite different from that reported by Korn et al (1965), see Table A.16 and Figure A.7A. When plasmodia were cultured on oats (the method initially used by Korn et al) the fatty acid composition was more comparable (see Table A.17 and Figure A.7B and A.7C). Microscopic observation of plasmodia grown on oats showed particles of oat streaming in the veins.

The age of the culture affected the fatty acid composition (see Table A.18). The material which changed most was that of relative retention time 0.88 (palmitate = 1.00). It decreased from 25% of the total acids at the maximum growth rate to 7% in resting cultures (sclerotia present). Oat grown plasmodia had only very low amounts of this material.

Qualitative differences in the fatty acid composition were observed between different plasmodial strains grown on agar (semi-defined medium) - see Table A.19 and Figure A.8. These differences in composition were later confirmed with plasmodia grown in liquid culture.

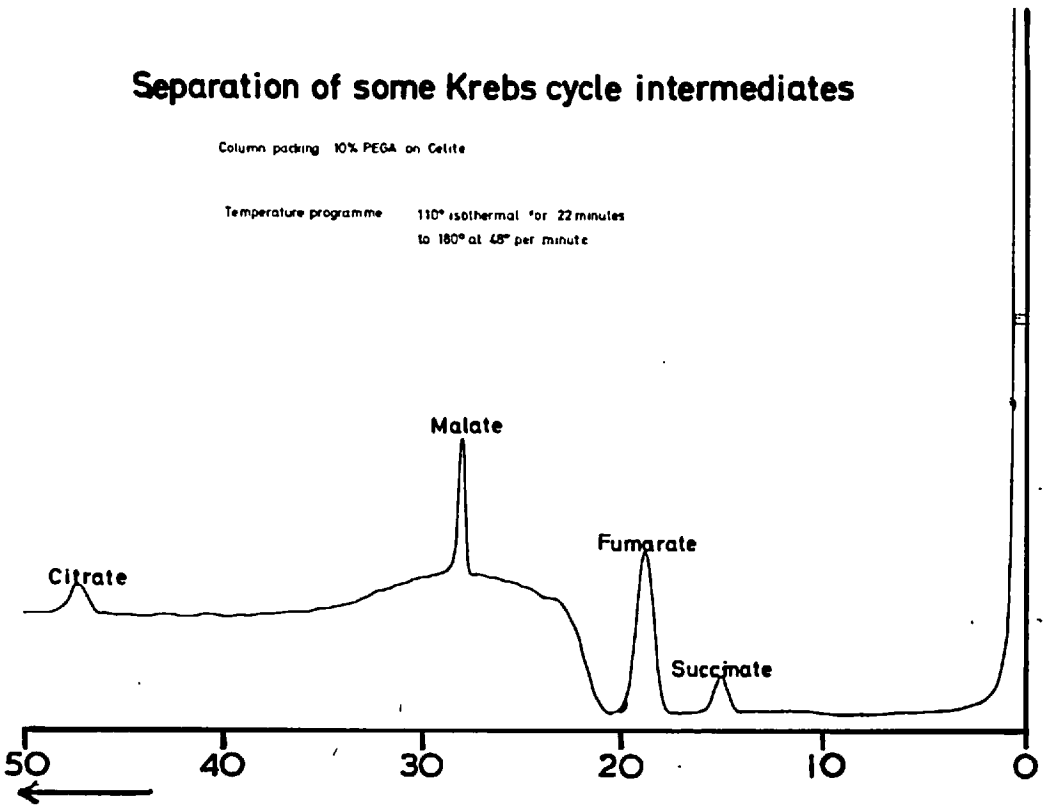
(contd. on p.294)

Separation of some Krebs cycle intermediates

Column packing 10% PEGA on Celite

Temperature programme 110° isothermal for 22 minutes
to 180° at 4.5° per minute

A



Krebs cycle intermediates from plasmodia

Column packing 10% PEGA on Celite

Temperature programme 110° isothermal for 22 minutes
to 180° at 4.8° per minute

B

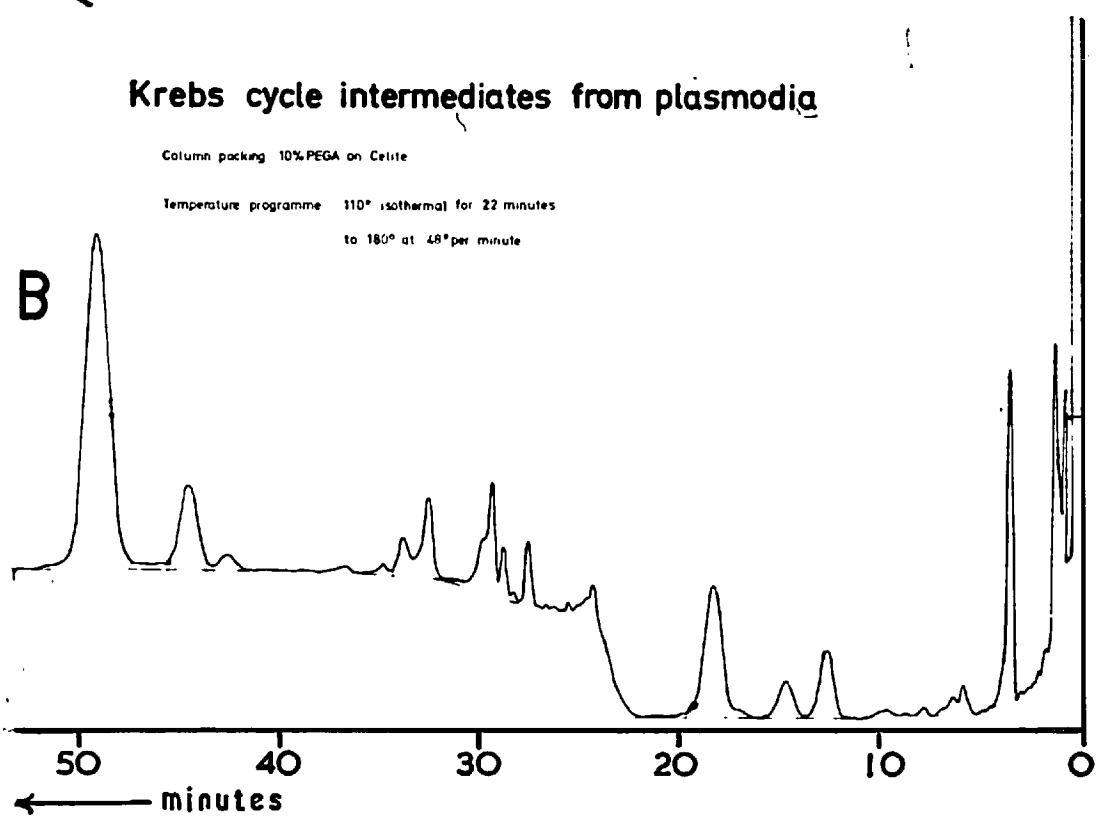


Figure A.6 The chromatographic separation of the methyl esters of the Krebs cycle intermediates

The methyl esters of the Krebs cycle intermediates derived from plasmodia (B) were prepared by a modification of the method of Barnett et al (1968) as described in Section A.2.d. The same method was used for the type compounds in A.

Gas chromatography was carried out on 10% PEGA on Chromosorb G using the temperature programme - 110°C isothermal for 22 minutes, 110-180° at 48°C per minute, 180°C isothermal for 30 minutes. Peak A was methyl lactate.

Table A.16

The fatty acid composition of plasmodia grown in standard medium

A 50 ml culture grown in standard medium was harvested by centrifugation (2000 g for 5 minutes) and added to chloroform-methanol (2:1 v/v). The lipids were extracted and converted to the methyl esters as described in Section A.2.e. Gas chromatography was carried out on 10% PEGA on Chromosorb G at 200°C.

Fatty acid	% of total	Korn et al (1965) data
Unknown R = 0.88	22.5	7.9
16.0 *	3.9	9.1
18.0	1.3	2.1
18.1	13.9	40.9
18.2	58.5	40.0

* Notation used for long chain fatty acids. The first number refers to the number of carbon atoms present, and the second the number of double bonds present.

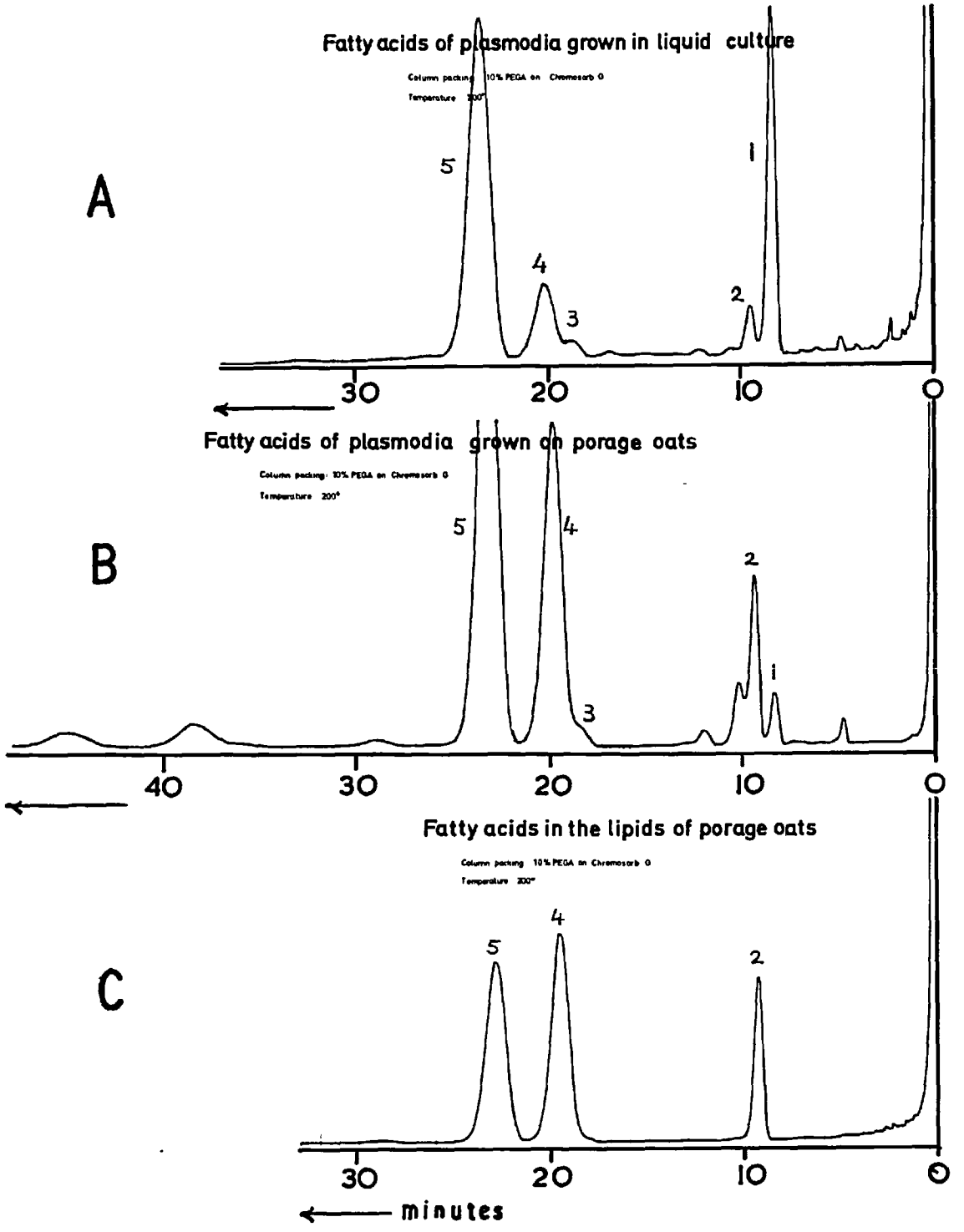

A
Fatty acids of plasmodia grown in liquid culture
Column packing 10% PEGA on Chromosorb G
Temperature 200°
5
1
4
2
3
30
20
10
0
B
Fatty acids of plasmodia grown on porage oats
Column packing 10% PEGA on Chromosorb G
Temperature 200°
5
4
2
i
3
40
30
20
10
0
C
Fatty acids in the lipids of porage oats
Column packing 10% PEGA on Chromosorb G
Temperature 200°
5
4
2
30
20
10
0
minutes

Figure A.7 Chromatograms of the total fatty acids and their methyl esters from plasmodia grown in standard medium and on porage oats, and from porage oats.

A Plasmodia (strain a + i) were grown in standard medium and harvested at near to maximum yield.

B Plasmodia (strain a + i) were grown on sterile oat flakes in a 500 ml Erlenmeyer flask and were harvested with a sterile knife when they had migrated up the wall of the flask clear of the oats.

C 1 g of oat flakes were taken and treated similarly to the plasmodia.

The method of extraction of the lipid and preparation of the methyl esters of the fatty acids is described in Section A.2.e. Chromatography was carried out on 10% PEGA on Chromosorb G at 200°C.

Peaks 1. unknown R_{RT} 0.88, 2. palmitate. 3. stearate.
4. oleate. 5. linoleate.

R_{RT} methyl palmitate = 1.00

Table A.17

The fatty acid composition* of plasmodia grown on oats

Plasmodia from liquid culture were inoculated on sterile oats in a flask (500 ml). After growth the plasmodia were allowed to migrate up the walls of the flask. They were removed with a sterile knife and extracted with chloroform-methanol (2:1, v/v). The methyl esters of the fatty acids were extracted and chromatographed as described in Section A.2.e.

Fatty acid	% of total	Korn et al data	Forage oats
Unknown R = 0.88	2.2	7.9	-
16.0	8.1	9.1	16.1
16.1	4.4	3.0	-
18.0	<1	2.1	-
18.1	31.8	40.9	42.3
18.2	54.1	40.0	41.7

* Omitting the contributions of minor peaks.

Table A.18

The change in fatty acid composition of plasmodia grown in standard medium with the age of the culture

Plasmodia (strain a + i) were harvested from cultures grown in semi-defined medium at the times indicated in the table. The lipids were extracted and the methyl esters of the fatty acids prepared as described in Section A.2.e.

Fatty acid	2% 4 day	5% 4 day	2% 7 day
Unknown	26.4	14.5	9.5
16.0	13.0	5.5	5.4
18.0	1.9	3.3	3.8
18.1	9.2	8.5	20.2
18.2	46.0	52.1	42.2
Others	3.7	12.8	18.6

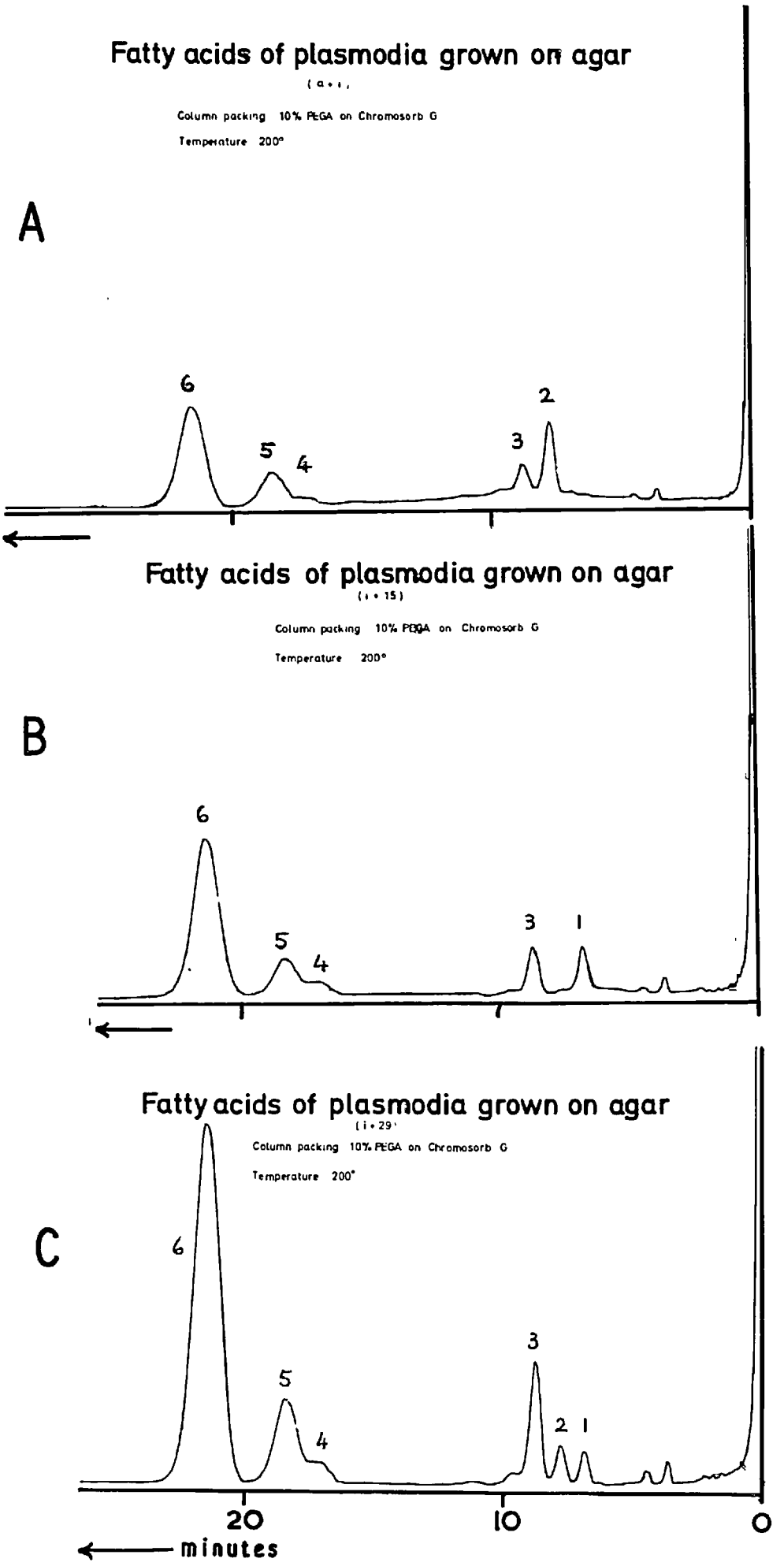

Fatty acids of plasmodia grown on agar
Column packing 10% PEGA on Chromosorb G
Temperature 200°
A
6
5
4
3
2
Fatty acids of plasmodia grown on agar
Column packing 10% PEGA on Chromosorb G
Temperature 200°
B
6
5
4
3
1
Fatty acids of plasmodia grown on agar
Column packing 10% PEGA on Chromosorb G
Temperature 200°
C
6
5
4
3
2
1
20
10
0
minutes

Figure A.8 The variation in fatty acid composition of different plasmodial strains when grown on agar.

Three strains of plasmodia a + i, i + A7015, i + A7029, were grown individually on agar plates (using the semi-defined medium described by Carlile, 1971). The plasmodia were floated off the agar when near to maximum yield, and their lipids extracted and the methyl ester of the fatty acids prepared as described in the Section A.2.e. Gas Chromatography was carried out on 10% PEGA on Chromosorb G at 200°C.

Peak identification:

1. Unknown R_{RT} 0.79 2. Unknown R_{RT} 0.89 3. Palmitate
4. Stearate 5. Oleate 6. Linoleate

R_{RT} methyl palmitate = 1.00

Table A.19

The fatty acid composition of different strains of plasmodia grown on agar

The plasmodial strains below were grown on semi-defined medium agar. The plasmodia were floated off and extracted with chloroform-methanol (2:1, v/v). Preparation of methyl esters of fatty acids and their gas chromatographic analysis was as described in Section A.2.e.

Fatty acid	a + i	i + A7015	i + A7029
Unknown R = 0.79	-	7.1	1.4
Unknown R = 0.89	16.8	-	2.0
16:0	5.4	8.9	9.8
18:0	< 1	< 1	3.1
18:1	16.5	12.9	13.4
18:2	61.6	71.0	70.1

The material of relative retention time 0.88 could be isolated by TLC of the methyl esters (using Kieselguhr G (nach stahl) impregnated with 10% silver nitrate: developed with petroleum ether 40-60°C, diethyl ether 9:1 v/v). The plates were sprayed with 2, 4-dichloro-fluoroscein (1% methanol solution) and the bands located under a U.V. lamp. The material ran between the saturated and mono-unsaturated methyl esters, being completely separated from both.

Discussion

1) Effect of nutrients on the growth of the plasmodium

a) Citric acid

Omission of citric acid had no effect on the dry weight yields at maximum growth (Table A.1, p.256). Citric acid was not metabolised by any of the strains tested (Table A.2 and A.3, pp. 257 & 258). Daniel & Rusch (1961) incorporated citric acid in their medium for the growth of P.polycephalum merely as a buffer (0.5 g/litre). That the total yield was not changed would confirm this.

For another myxomycete, Physarum rigidum, Henney & Henney (1968) found that in their medium citric acid was toxic at a concentration of 5 g/litre, whereas the levels of citric acid in the semi-defined medium (Carlile, 1971) used in this study (3.5 g/litre) permitted active growth of P. polycephalum.

b) Glucose

A value of 25-35 mg glucose/g dry weight/hour was consistently obtained for plasmodia incubated aerobically in standard medium containing various levels of glucose. The rate was not affected by transfer to standard medium lacking peptone (Table A.4, p.260). The consistent level of glucose uptake was unlike animal muscle systems where the rate of uptake was dependent on the external glucose concentration (Park, 1955; Mansford, 1969). A possible explanation for this is discussed later.

2) Storage compounds of the plasmodium

a) Glycogen

Several workers have described the internal polysaccharide as glycogen or glycogen-like. Sullivan (1953) reported a decrease in the glycogen content (% of the dry weight) in sclerotia compared with plasmodia. Daniel (1964) isolated a polysaccharide by the KOH-ethanol glycogen isolation procedure and found it to consist of glucose and to stain reddish-brown with iodine. Goodman & Rusch (1969) isolated a polysaccharide by the procedure of Orrell et al (1964). The effect of α-amylase and β-amylase in partly degrading the polysaccharide was demonstrated by electron microscopy and was said to be similar to that observed using rabbit liver glycogen. Also the spectrum of the polysaccharide-iodine complex was similar to that obtained with rabbit liver glycogen.

In this study the polysaccharide could be resolved into two fractions, one readily soluble in water. The other polysaccharide was thought to be the slime released into the medium. This material has been shown to be a galactose polymer (Simon & Henney, 1970). This had been previously observed in this laboratory (Buck, K.W. - personal communication).

Evidence in this study that the water soluble polysaccharide was similar to other glycogens was its precipitation by low levels of ethanol, the spectrum of the complex with iodine (Figure A.1, p.264), its glucose content and its degradation by α-amylase and β-amylase. Exhaustive degradation with β-amylase released 45% of the total glucose, a value corresponding to that observed with other glycogens which were degraded at the same time.

b) Fatty acid composition

The fatty acid composition of plasmodia grown in standard medium in liquid culture and on agar was different from that obtained for plasmodia grown on porage oats (Tables A.16 and A.17, pp. 286 + 289 and Figure A.7, p. 288). It was also different from that reported by Korn et al (1965) for plasmodia grown in liquid culture (Tables A.16 and A.17). The values reported by Korn et al (1965) for palmitic, oleic and linoleic acids were similar to those obtained for the fatty acids of porage oats (Table A.17). Their results might be explained in terms of the age of the culture examined, an increase in the oleic acid content was observed in older cultures (post maximum growth) - see Table A.18, p. 290.

The large difference in percentage composition of the material of relative retention time 0.88 (palmitate = 1.00) measured in this study and in that of Korn et al (1965) may well be due to genetic differences between the plasmodia used. As shown in Table A.19, p. 293 in strain i + A7015 this material was not present at all and in strain i + A7029, was present in smaller amounts. Thus with plasmodia of mixed genetic pairs the level would depend on the composition of the different strains present. By observing the presence or absence of this material and also that of relative retention time 0.79 it was possible to distinguish between strains. The nature of both these materials was not investigated although Korn et al (1965) suggested they were branched fatty acids.

3) Synthetic and metabolic pathways

a) Glycogen synthesis

Plasmodia from the active growth phase had high levels of glycogen and when they were incubated with glucose although synthesis

did take place the net increase was a small percentage of the glycogen initially present (see Table A.7, p.267). Incubations with glucose labelled with [U-^{14}C]-glucose indicated that 60-70% of the glucose which disappeared from the medium was converted to glycogen despite the small net increase in the mass of glycogen.

Glycogen-depleted plasmodia were used to examine the synthesis more closely. A large net synthesis of glycogen was observed during the incubation in parallel with the incorporation of ^{14}C-glucose (Table A.8, A.9 and A.10, pp.268,271+274). A time course study of the synthesis showed that in the first 15 minutes 100% of the ^{14}C-glucose which disappeared from the medium was incorporated into glycogen (Table A.10) and at the same time 60% of glucose used up appeared as the net increase in the level of glycogen. The difference, 40% of the glucose taken up, represented the endogenous glycogen metabolised during this period. If the specific radioactivity of the increment during incubation was calculated it would be higher than that of the substrate glucose. This situation has been observed during glucose transport across the small intestine of the rat (Porteous & Pritchard, 1972). This would seem to indicate that glucose was first converted to glycogen which was later broken down. The most recently synthesised glycogen was not the immediate substrate for breakdown since all the radioactivity remained as glycogen in this first period. As the time course continued the percentage of ^{14}C-glucose incorporated into glycogen decreased as did the percentage of glucose appearing as a net increase of glycogen. There was no difference in incorporation between [1-^{14}C]-glucose and [2-^{14}C]-glucose and [6-^{14}C]-glucose (Table A.9). This would also suggest that direct synthesis to glycogen has occurred since the pentose phosphate shunt was operative in plasmodia and this would be expected to affect the specific activity of G6P if labelled in the C-1 position

compared with the C-6 position (Katz & Wood, 1960). Since G6P is generally accepted as being a precursor of glycogen then the specific activity of the glycogen would have been expected to be different (less for C-1 labelled G6P).

With glycogen-depleted plasmodia uptake of 15-20 mg of ^{14}C-labelled glucose by the plasmodia was sufficient to result in the glycogen having the same specific activity as the glucose (see Figure A.3, p.273). Immediately after the disappearance of the external glucose the level of glycogen began to drop, the specific activity remaining constant. Judging from the continued incorporation of ^{14}C-activity into lipid (see Figure A.5, p.282) despite the lack of external glucose, glycogen was a substrate for metabolic processes. Studies on the aerobic respiration of plasmodia indicated that for plasmodia from the active phase of growth the oxygen uptake and carbon dioxide production were not affected by the absence of substrates, either glucose or peptone, during the course of the experiment (one hour).

b) Glycolysis

Evidence for glycolysis has come as a result of the study of anaerobiosis and the motive force of streaming. The energy for the motive force is considered to be ATP (Kamiya, 1959). He found that lowering of the oxygen tension increased the motive force, as did any condition which suppressed respiration. From this he concluded that an increase in the rate of glycolysis had occurred due to the Pasteur effect. Ohta (1954) also reported the Pasteur effect, the amount of an acid (not identified) produced by plasmodia in anaerobic condition, being five times as high as that produced in aerobic conditions.

Hatano & Takeuchi (1960) measured the plasmodia ATP levels and found that they were not changed by low oxygen tensions. If the ATP produced in the mitochondria was decreased by low oxygen tensions then that produced by glycolysis would be increased, causing an increase in the motive force as observed by Kamiya.

Several workers have reported that plasmodia could continue to stream under anaerobic conditions (Seifriz & Urbach, 1944; Loewy, 1950; Allen & Price, 1950). Kitching & Pirenne (1940) however reported that complete removal of oxygen caused rapid cessation of streaming. Recent work of Carlile (1970 - private communication) with axenic material has shown that in the complete absence of oxygen cessation of streaming was rapid, and that 95% nitrogen/5% carbon dioxide gas mixture could not extend the streaming period to 24 hours as reported by Loewy (1950). Oxygen impurities in the nitrogen and carbon dioxide used in previous studies may well have accounted for the continued streaming in apparently anaerobic conditions. Thus despite these differences of opinion it was very likely that glycolysis occurred in plasmodia, was stimulated by lowering the oxygen tension and was inhibited by total lack of oxygen. The presence of G6P, F6P and lactate as determined by GLC gives further evidence for glycolysis.

It has been suggested (Gray & Alexopoulos, 1968) that ATP generated by glycolysis was used as the energy source for streaming rather than ATP generated by oxidative processes, since glycolysis takes place in the groundplasm where the motive force appears to be generated. It has not been demonstrated whether ATP generated in mitochondria could diffuse readily into the groundplasm.

c) The pentose phosphate shunt

Since synchronised surges in DNA synthesis occur in the plasmodium (Rusch, 1969) it seemed likely that the pentose phosphate shunt was in operation to provide the necessary substrates. In addition Hüttermann (1969) has shown that glucose-6-phosphate dehydrogenase increases during the growth phase.

When measurements were made of the $^{14}CO_2$ released after incubation with [1-^{14}C]-glucose and [6-^{14}C]-glucose that despite the variability of the results it was clear that [1-^{14}C]- glucose was converted to $^{14}CO_2$ more readily than was [6-^{14}C]-glucose (see Table A.12, p. 277 and Figure A.4, p.279). Increased incorporation of ^{14}C-activity into the lipid fraction was observed during incubation with [6-^{14}C]-glucose than from [1-^{14}C]-glucose (see Table A.13, p.280 and Figure A.5, p.282). For active non-starved plasmodia the ratio of incorporation of C-6 labelled activity and C-1 labelled activity was 3:2, whereas for starved plasmodia it was 2:1 although the total incorporation was reduced. Both of these observations indicated strongly that the pentose phosphate shunt was operative in aerobic active plasmodia.

However, it was not possible to calculate the contribution of the shunt to the total metabolism by the methods discussed by Katz & Wood (1960, 1963). The large amount of glycogen synthesis ruled out most theories, and for those developed for a situation where glycogen synthesis was high, the qualitative difference in metabolism for this organism compared with those discussed by Katz & Wood (1963) invalidated them.

Examination of the activities of the intermediates isolated after incubation with [1-^{14}C]-glucose and [6-^{14}C]-glucose by radio-GLC of their TMS-derivatives (see Figure 7.3, p.190) indicated that the

Krebs cycle intermediates were labelled more from [6-^{14}C]-glucose than from [1-^{14}C]-glucose (see Table 7.3, p.191). This is in agreement with that observed for incorporation in lipid.

d) The Krebs cycle

The presence of mitochondria has been reported by many workers. Dugas & Bath (1962) using electron microscope studies described the mitochondria as resembling those of most protozoa and other simple organisms. Holter & Pollock (1952) reported that the enzyme succinic dehydrogenase was located in the mitochondrial fraction. Johnson & Moos (1956) reported similarly for the enzyme succinic oxidase.

Ohta (1954) reported that the $Q_{CO_2}^{N_2}$ for plasmodia was one-eighth that of $Q_{CO_2}^{air}$ indicating that aerobic metabolism was responsible for most of the carbon dioxide evolved by plasmodia. The presence of some of the Krebs cycle intermediates as determined by GLC (see Figure A.6, p.285 and Figure 7.3, p.190) with the previously mentioned observation would suggest, although not prove, the existence of the Krebs cycle in plasmodia.

4) Formation of sclerotia

Incubation of plasmodia in standard medium lacking peptone and glucose led to the rapid formation of sclerotia within 24 hours (see Table A.5, p.261). McCormick & Rusch (1969) have also observed this effect. Chet & Rusch (1969) have caused formation of sclerotia from plasmodia in normal growth medium by the addition of sugar alcohols, mannitol (to 0.5 Molar) being the most effective. Jump (1954) has reviewed the situations which caused the formation of sclerotia.

One of these situations was exhaustion of nutrients. The composition of the growth medium used in this study was chosen so that both the nitrogen source, the peptone, and the carbohydrate source, the glucose, were both nearly zero at maximum growth (Carlile, 1971). For cultures grown on this medium, formation of sclerotia occurred routinely two or three days after maximum growth. It was not clear whether lack of either the peptone or the glucose, or both, was responsible for the formation of sclerotia.

When plasmodia were incubated in standard medium lacking glucose formation of sclerotia was almost entirely prevented whereas incubation in standard medium lacking peptone resulted in greater than 70% of the plasmodia becoming sclerotia (see Table A.5, p.261). Thus the presence of high levels of glucose could not prevent formation of sclerotia.

Examination of the glycogen levels (Table A.6, p.265) showed that the level did not control onset of the formation of sclerotia. Plasmodia incubated in standard medium lacking glucose had a very low level of glycogen whereas those incubated in standard medium lacking glucose and peptone had a higher level despite total conversion to sclerotia. Those incubated in the presence of glucose (1%) maintained the level of glycogen.

Thus lack of glycogen did not significantly alter the state of the plasmodia. Continued streaming in standard medium lacking glucose for longer periods than that reported in this study have been observed (M.J. Carlile - private communication). In this circumstance it was likely that glycogen was depleted to provide the energy for streaming and then other energy was provided by the oxidation of amino

acids from the peptone. The myxoamoebae of the cellular slime mould, Dictyostelium discoideum, grow adequately in axenic culture on peptone alone (Watts & Ashworth, 1970) and addition of glucose suppressed the oxidation of amino acids without significantly enhancing growth. For plasmodia of P. polycephalum growth on peptone does not occur.

In the biological situation although onset of the formation of sclerotia is rapid in the absence of nutrients there is a sufficient store of energy for the plasmodium to search for new sources of food before doing so. Carlile (1971) has shown that plasmodia can detect food concentration gradients and migrate up them. When a food source is found the starved plasmodia appears to convert the carbohydrate to glycogen first of all, so that if the source were limited it does not waste the food by using it for growth. Thus it is able to move off to find another site. In this state the rate of breakdown of glycogen would be governed by its level, providing other necessary substrates were present. If this is the case then the rate of uptake of glucose would not be affected by the remainder of the metabolism since starved plasmodia can accommodate large amounts of glycogen. It may be postulated that the rate of glucose uptake was similar to the rate of glycogen breakdown at maximum glycogen levels (in 20% of the dry weight). Whether an entirely new method of glycogen synthesis is operative in this organism or whether discrete compartmentilisation of the sites of synthesis occurs remains to be investigated.

APPENDIX 2

COMPUTER PROCESSING OF GAS LIQUID CHROMATOGRAPHY DATA

1) Introduction

The complexity of the chromatograms obtained when analysing tissue samples, and the need to be able to determine quantitative data necessitates automation of the data analysis.

Integration of peak areas can be carried out manually (e.g. by peak triangulation), by the use of integrators (mechanically or electronically), or by using a computer. The disadvantage of the manual method is the amount of labour necessary. The disadvantage of integrators which work in real time (i.e. as the data is being produced) is that for the situation where the baseline is changing no allowance can be made for baseline change during the peak. Also the integrator (i.e. non-computing) cannot analyse the data to correct for the area of the standard peak, or to attempt to identify the peaks according to their retention time. This type of machine is useful only for essentially simple chromatography where the results are not urgently required.

To be able to analyse the data more thoroughly a computer is necessary. The definition of a computer in this context is an instrument which has the ability to store information (this includes preprogrammed information) and has the ability to marshal information, and selectively produce the required data. Two types of computer can be considered. Firstly, the on-line system; this works essentially in real time and therefore is subject to certain limitations concerning baseline changes.

In some cases the raw data from the chromatograph is fed direct to the computer. However, this is very wasteful of computing time, and so a small satellite computer which processes the information is often used. With such a system many analyses from different machines can be handled by the central computer at once. The central computer is large and therefore can carry out complex analyses on the data, or can be used to impose its control on a process. Such systems are most commonly found in industry.

For users with small quantities of complex chromatograms the off-line computer is more economic. In the case of the off-line computer the data from the chromatogram is recorded either in an analogue or, more usually, a digital form, with a secondary system, and the data presented to the computer at a later time. This could of course be carried out by a satellite computer as mentioned above. The normal methods include recording onto punched cards, onto paper tape, or onto magnetic tape. The particular advantage of these methods is that the raw data is recorded in a permanent form which is not the case when using the on-line computer.

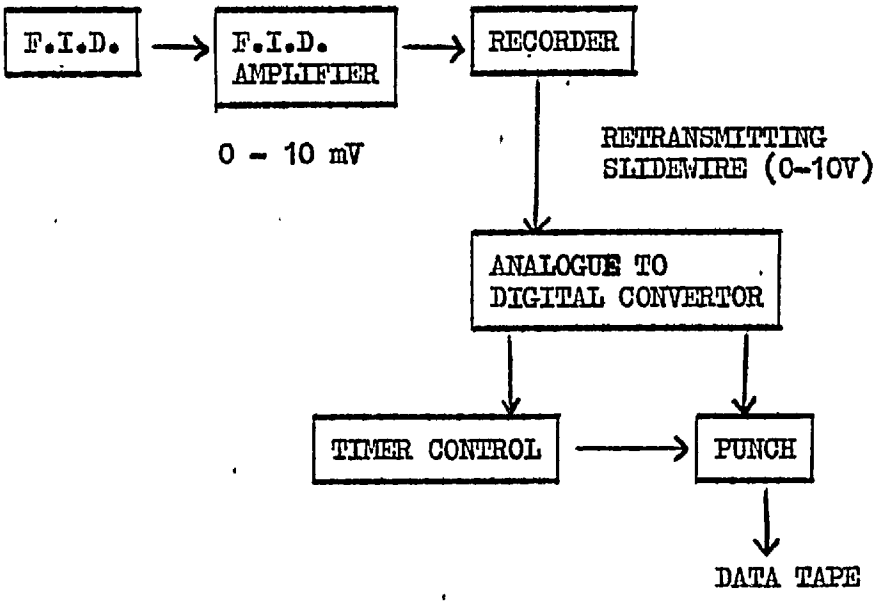
In this study recording onto paper tape was used. This was less cumbersome than cards, but not as convenient as magnetic tape which can be fed into the computer more rapidly than the other two. This is an important consideration since the major portion of the computing time, and hence costs, is spent feeding in and printing out information.

The data recorded onto the paper tape was in the binary decimal form and samples were taken per unit time. There was no need to record the data at speeds greater than one data point per second

for the relatively low speeds of the chromatography used in this study. The minimum number of data points needed to describe a peak with an error of less than 0.1% were 13 (Kishimoto & Musha, 1971); this criterion was fulfilled for the peaks examined.

2) <u>The recording of the output of the chromatograph onto punched tape</u>

The arrangement for recording the data was as is outlined below:



The output of the F.I.D. amplifier (0-10 mV) was not sufficient to drive the analogue to digital convertor, so the recorder (Kent Mark III) was fitted with a retransmitting slide wire. This slidewire was parallel to the slidewire used for balancing the input from the F.I.D. amplifier. This extra slidewire had a potential drop across it of 10V. The carriage on the servo arm had two pick ups, one for each wire, so that the corresponding position on the 0-10 mV slidewire was tapped off on the 0-10V slidewire. This results in a 1000 fold amplification. In fact it was arranged that the bottom end of the

slidewire still registered a slight voltage (0.07V), this preventing the punching of 000 when operating normally. Similarly the top end of the wire only registered 9.80V, this preventing 1000 or greater being punched (a four figure number).

This analogue signal was converted to a binary decimal form, this then being punched out (Addo Tape punch) under the control of a timer unit (built in the electronic workshop of the Biochemistry Department, Imperial College, by A.E. Lowe).

At the beginning of the analysis the time constant was punched onto the tape (by a button on the timer unit). This data block also had a minus (-) sign in the first line. This identified the beginning of the data. At the end of the data tape, or when a baseline or sensitivity change was necessary, another minus sign was punched. One of the read instructions for the computer included identification of the first line of the data block. If a minus sign was not punched when altering the conditions of the analysis (e.g. baseline or sensitivity change) manual editing was carried out. After changing the conditions another minus sign plus time constant was punched. The computer programme deal with the data in each section as a distinct entity as far as area measurement was concerned, but it did accumulate the total time since the beginning of the analysis. A job terminator was attached to the end of the tape after a final minus sign.

3) <u>The computer programmes</u>

The programmes which have been written for the integration of peaks have not dealt with situations where the baseline changes rapidly. The programmes used in this study have been written specially for this, although they can be used for any chromatographic analysis.

Two computer programmes have been used; one in Algol with the London University Atlas computer, and the other in Fortran with the PDP-9 computer. Essentially the mathematical processes in both programmes were the same. The differences were in the manipulation of these processes necessary because of the different sizes of store available with each computer.

a) The Algol Programme

Imperial College had a direct link to the Atlas computer and this was used initially. This computer has a very large store and enabled the operator to obtain fast execution with the expense of using up more store than was economic. To check the execution of the programme it was necessary to output several of the arrays generated (e.g. baseline and corrected data). This was a costly operation and interpretation of the large amount of digital information was not easy for the observer. Because of this the PDP-9 computer which could display this data in an analogue form on a television screen was used.

Since the final programme was developed from that written for the Atlas computer an outline of the steps of the initial programme will be described in this section.

A. Input of data

The data was read in from the tape, discarding any points of value 0 or greater than 1000. Any parity or format errors brought input to a halt. The tape had to be restarted after correction of errors.

B. Removal of spikes

Spikes due to dust passing through the flame ionisation detector were removed by averaging each point either side of the data point examined and comparing with the data point. If it was a spike

the value was replaced by the average of the points either side.

C. Smoothing of the data

The data was passed through a smoothing routine until the conditions for smoothed data were achieved. The number of iterations was recorded.

D. Calculation of the 1st, 2nd and 3rd differentials of the data

These values were stored in three separate arrays.

E. Determination of baseline points

The following conditions had to be satisfied:

1) 1st differential must be > 1st differential found at the last baseline point (set at an initial value of -1000).
2) 1st differential must be < 0
3) 2nd differential must be = 0
4) 3rd differential must be = 0

It was assumed that baseline slope either became less negative, or remained constant throughout the analysis (conditions 1 & 2). Condition 3 eliminated tops of peaks from being recognised as baseline. Condition 4 overcame the problem of apparent small valleys between peaks caused by variation of the data at the top of wide peaks. The first and last data points were also taken as baseline points. The punching of the tape was begun once the solvent front had reached the detector. this meant that the first data point read 9.80.

F. Construction of baseline

The established baseline points were joined together linearly, updating the baseline formed where the data was less than the baseline.

G. Subtration of baseline from smoothed data

This isolated the peaks to give 'pure' chromatography.

H. Calculation of data

The programme proceeded through the 'pure' data until it noticed the start of peak. Integration was begun and continued until the top of the peak was found. This height was recorded and the integration continued either until the baseline (zero) was found or the valley between two peaks. If there was a valley the total area up to the lowest point of the valley was recorded and integration restarted through the peak maximum and down to zero. When baseline (zero) was found the programme proceeded through the data until it found the beginning of the next peak.

I. Output of data

The integrated area of each peak was multiplied by the time interval between data points operative in that part of the data, and printed. The peak heights for each peak were printed and also the time from the beginning of the analysis (essentially retention time). In addition the contents of the arrays holding the smoothed data, baseline points, calculated baseline and purified data were printed so as to check the execution of the programme.

b) The Fortran Programme

This was written for use with a PDP-9 computer which had a limited store (8K) compared with the Atlas computer and was an adaption of the first programme. With the Atlas computer a separate array was used for the input data, smoothed data, 1st, 2nd and 3rd differentials, baseline points, baseline and 'pure' data. With the PDP-9 this number of arrays was not possible and a running method of analysis was used.

The amount of store space left after entry of the programme permitted 720 data points to be analysed. Since the GLC analysis took 75 minutes and the punching rate was 20 blocks per minute it would be impossible to analyse the entire data tape in one go. However, the

analysis was broken into sections caused by changes in sensitivity so that 720 was not always exceeded. In addition parity and format faults did not bring input to a halt. They were recorded by output on the line printer.

Essentially the same mathematical procedures were adopted as outlined for the Algol programme. Additional procedures were incorporated into the programme. The PDP-9 has video-display facilities and it was possible to display both the smoothed data and the calculated baseline. The programme was halted at this point whilst the operator examined the baseline. If the baseline was satisfactory a key was lifted on the switch register, restarting the programme and printing out the integrated data. In many cases either the data was still very rough or the operators concept of where the baseline should be and that calculated by the programme were different. This often meant baseline points being found on downward slopes before 'true' baseline had begun. These were readily improved by resmoothing the data, the signal for this being given by lifting another key on the switch register.

In the second and third sections of a data tape the definition of baseline had to be modified. In some cases these sections began with a rising peak, no baseline points being found before the top of the peak. On the downward slope baseline was found very rapidly since all the conditions (E. 1-4) were satisfied. This was overcome by resetting the 1st differential (condition E.1) to be initially - 2. A baseline point could not be found until the baseline was almost reached. This modification was only carried out if an anomalous position of the baseline at the start of the section was observed. Again it was effected by lifting the appropriate key on the switch register. The layout of this programme is shown in Figure A.9.

c) Organisation of the Fortran programme

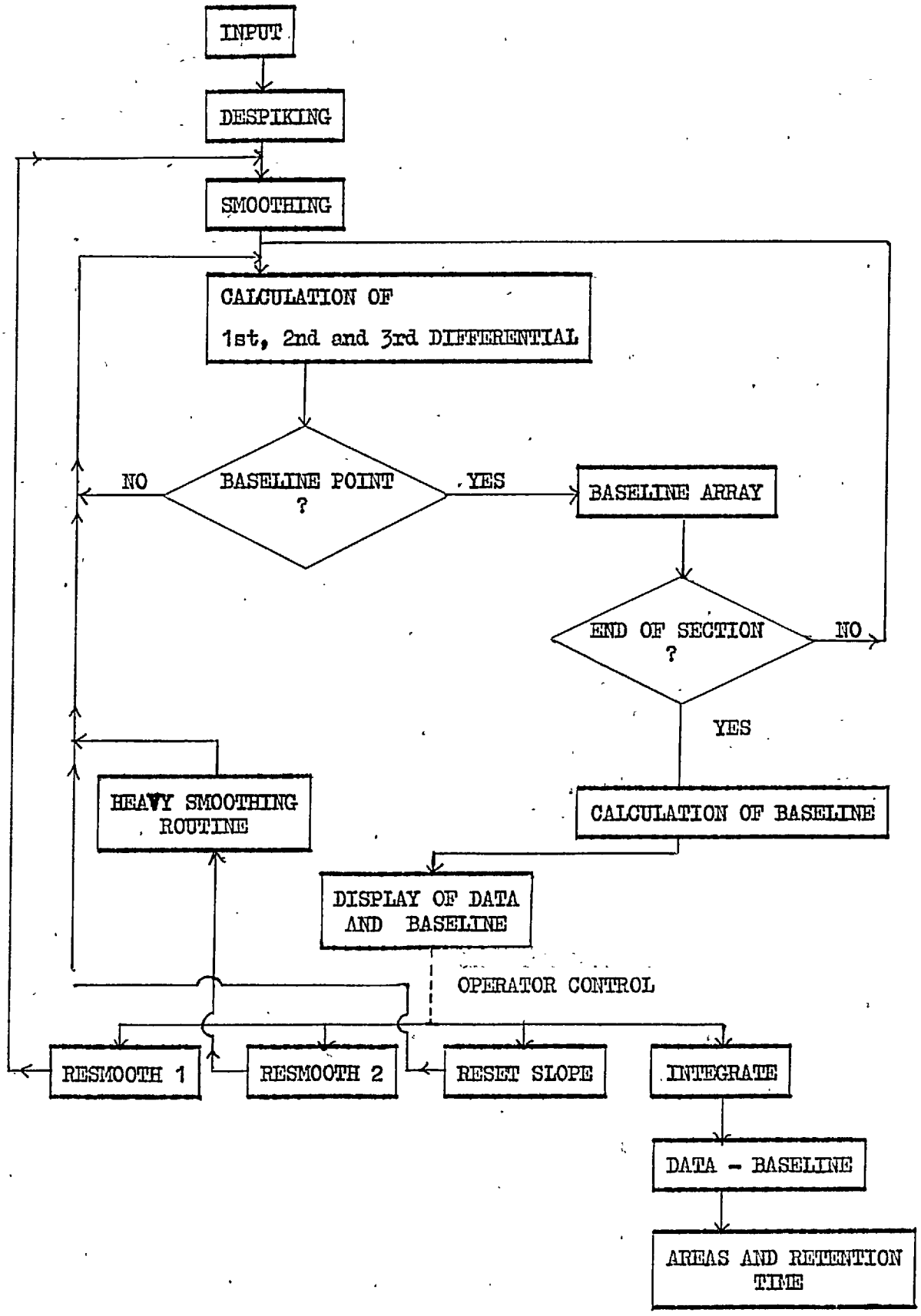
Two arrays were used. The input data was fed into array 1. After despiking the data was smoothed, each smoothed data point being put in the corresponding space in array 2. Instead of generating arrays for 1st, 2nd and 3rd differentials, each point was taken in turn and the 1st, 2nd and 3rd differentials for that point calculated. The point was checked to see if it satisfied the baseline conditions or not. If it did the corresponding data point in array 1 was replaced by a 1. If it did not, a zero (0) was inserted. When all the points had been examined all the smoothed data point values in array 2 with a 1 in the corresponding position in array 1 were made negative. The negative numbers were joined linearly and the extrapolated baseline transferred to array 1 (now positive). Each of the intermediate points were checked to see that the baseline was not greater than that point. When the baseline was finally ready both it and the smoothed data were projected on the screen. Resmoothing caused the values in array 2 to replace those in array 1 and the whole procedure to be repeated, as if it were fresh data. Changes of the slope condition caused the programme to be restarted at the assessment of each point as baseline.

When the operator was satisfied with the baseline the data in array 1 was replaced by the difference between array 2 and array 1 and hence integration as described for the Algol programme was started.

4) Discussion

The operator-computer interaction which was possible with the PDP-9 was a major advantage over the complete lack of control using the Atlas computer. The operator was able to correct most of the

Figure A.9 Layout of the programme used with PDP-9 computer



fallacies which were caused by the programme. In the early stages of the development of the programme it was envisaged that the operator could draw in the baseline for the data which was displayed on a screen, by using a light pen. However, human error and bias was possible and so this level of interaction was not pursued.

Occasionally mistakes, generally caused by faulty punching of the data, occurred. This meant that data points were displayed in anomalous positions, causing disruption of baseline calculations. This may be overcome by the use of a moving spot. The spot could be scanned across the screen until it corresponded to the abcissa of the faulty data; the operator could then type in the value which he thought was the correct one.

Many GLC-computer applications had been reported (see Childs et al, 1969; Kuzel et al, 1969). The majority have been concerned with on-line systems calculating routine GLC analyses. In on-line systems the output from the detection system was fed directly to the computer in real time and was not stored in a permanent form. On-line systems have advantages over off-line systems in that the data produced was more rapidly available, in many cases sufficiently quickly to control plant processes.

However, for non-routine analyses the speed of availability of data is generally not important. Oberholtzer (1969) has examined the use of off-line systems, and although agreeing to their usefulness for non-routine analyses criticises the accuracy of the data produced, particularly the baseline. However, the inaccuracy of the data was very small compared with the other errors including experimental errors. Since baseline by the current procedure was accurately determined, the errors discussed by Oberholtzer, largely inaccuracies of the baseline,

are removed. Since on-line systems are very expensive no justification can be seen for use with experimental analyses.

A serious disadvantage of on-line systems is the lack of permanent record of the chromatographic data. Computer failure and less serious hiccups are not uncommon. With a permanent record recalculation of the data can be carried out if programme alterations were necessary at a later date. Although paper tape was used in this study, magnetic tape is a better proposition since the data can be recorded faster and stored in a much smaller space.

A disadvantage of the current system was the limited range of the potentiometric recorder. The baseline at the lowest attenuation range used (x 100) was still essentially noise-free. Spikes caused by dust particles passing through the flame ionisation detector occurred only to a limited extent and were filtered out by the programme. Thus the baseline noise would still be reasonable at x20 and in any case noise is averaged out by the data sampling. However, the drift of the baseline would be prohibitive with the arrangement described in this study.

The potentiometric recorder was limited to reading 0-10 mV, the analogue to digital converter effectively reading this in 10 μV steps. If the recorder and the analogue to digital converter were replaced by a five decade digital voltmeter with automatic range changing almost any value could be kept on scale. The range 0-100 mV could be used still reading data in 10 μV. The range needed to keep the data on-scale would be punched onto the data tape and the data multiplied by this value once in the computer. This would greatly enhance the usefulness of the system since an operator would no longer be required

to intervene and change the attenuation range during the analyses. With the PDP-9 each word has 18 bits. This means that the greatest value of the data in a single word would be limited to $2^{17} - 1$ (131,071). The data could be entered as two words but this is a problem since the amount of data that can be handled by the program is 720 data points. Increasing the store by 2K more words would overcome this.

COMPUTER PROGRAMME USED WITH THE PDP-9 COMPUTER

```
      DIMENSION IBUFF(1ØØØ), IDATA(725,2)
      COMMON IBUFF,IDATA,ITIME,INT,ISIG
 5    ITIME=Ø
 7    CALL TAPE(ISIG,INTA)
 8    WRITE(1,1ØØ1)INTA
      INT=INTA/1ØØ
1Ø    NPOINT=1
11    CALL TAPE(ISIG,IDATA(NPOINT,1))
      IF (ISIG.EQ.1) GO TO 16
      IF (ISIG.EQ.2) GO TO 13
      WRITE(1,1ØØ1)NPOINT
      GO TO 11
13    IF(IDATA(1,1).GT.94Ø)GO TO 11
      IF(IDATA(NPOINT,1).EQ.Ø)GO TO 11
      IF(NPOINT.EQ.725)GO TO 16
15    NPOINT=NPOINT+1
      GO TO 11
16    NPOINT=NPOINT-2
      DO 2Ø N=2,NPOINT
      IDA=(IDATA(N-1,1)+IDATA(N+1,1))/2
      IF(IDATA(N,1).GT.IDA+3Ø) IDATA(N,1)=IDA
2Ø    CONTINUE
22    DO 25 N=2,NPOINT
      IDATA(N,2)=(IDATA(N-1,1)+IDATA(N,1)+IDATA(N+1,1))/3
25    CONTINUE
      IDATA(1,2)=IDATA(1,1)
      ID1LAS=-1ØØØ
      NPOINT=NPOINT-3
```

```
26      DO 3Ø N=2,NPOINT
        IDATA(N,1)=Ø
        ID1=ID2
        ID2=ID3
        ID3=ID4
        ID5=ID6
        ID4=IDATA(N+3,2)-IDATA(N+2,2)
        ID6=(ID4+ID2)/2
        ID7=ID6-ID5
        IF(IDATA(N,2).GT.95Ø)GO TO 3Ø
        IF(N.LT.21)GO TO 27
        IF(IDATA(N,2).GT.IDATA(N-1Ø,2)+2)GO TO 3Ø
        IF(IDATA(N,2).GT.IDATA(N-2Ø,2)+5)GO TO 3Ø
27      IF(ID2.GT.Ø)GO TO 3Ø
        IF(ID2.LT.ID1LAS)GO TO 3Ø
        IF(ID5.NE.Ø)GO TO 3Ø
        IF(ID7.NE.Ø)GO TO 3Ø
        IDATA(N,1)=N
        ID1LAS=ID2
30      CONTINUE
        DO 35 N=1,NPOINT
        IF(IDATA(N,1).GT.Ø)IDATA(N,2)=-IDATA(N,2)
35      CONTINUE
        DO 5Ø N=1,NPOINT
        IDATA(N,1)=-IDATA(N,2)
        IF(IDATA(N,2).LT.Ø)GO TO 5Ø
        IF(IDATA(N-1,2).GT.Ø) GO TO 4Ø
        IJ=N-1
        IX=-IDATA(IJ,2)
        IK=N
```

```
37      IK=IK+1
        IF(IDATA(IK,2).GT.Ø) GO TO 37
        IY=-IDATA(IK,2)
4Ø      IDATA(N,1)=(((IX-IY)*(IK-N))/(IK-IJ))+IY
        IMIN=IDATA(N,2)-IDATA(N,1)
        IF(IMIN.GT.Ø) GO TO 5Ø
        NFIN=K-1
        DO 45 M=N,NFIN
        INEX=IDATA(M,2)-IY-(((IX-IY)*(IK-M))/(IK-IJ))
        IF(INEX.GT.IMIN)GO TO 45
        IMIN=INEX
        N=M
45      CONTINUE
        IDATA(N,2)=-IDATA(N,2)
        N=IJ
5Ø      CONTINUE
51      NP=((NPOINT+2)/3)*4
        CALL SCALE(NP,Ø.Ø.75Ø,Ø,Ø,Ø,Ø,1ØØØ,Ø,IBUFF)
        DO 52 N=1,NPOINT
        IF (IDATA(N,2).LT.Ø) IDATA(N,2)=-IDATA(N,2)
52      CONTINUE
        DO 53 N=1,NPOINT,3
        R=N
        CALL DSPLY(R)
        R=IDATA(N,2)
        CALL DSPLY(R)
53      CONTINUE
        CALL ENDPT
        DO 54 N=1,NPOINT,3
        R=N
```

```
      CALL DSPLY(R)
      R=IDATA(N,1)
      CALL DSPLY(R)
54    CONTINUE
55    CALL ACSW(ISIG)
      IF(ISIG.EQ.Ø)GO TO 55
      IF(ISIG.EQ.1)GO TO 9Ø
      IF(ISIG.EQ.2)GO TO 95
      IF(ISIG.EQ.4)GO TO 82
      IF(ISIG.EQ.8)GO TO 11Ø
      WRITE(1,1ØØ3)
      ID1=Ø
      DO 8Ø N=1,NPOINT
      IDATA(N,1)=IDATA(N,2)-IDATA(N,1)
      IF(ID1.NE.Ø)GO TO 58
      IF(IDATA(N,1).LT.2)GO TO 8Ø
56    ID1=1
      ID2=IDATA(N,1)
      GO TO 8Ø
58    IF(ID1.EQ.Ø-1) GO TO 62
      ID2=ID2+IDATA(N,1)
      IF(IDATA(N,1).GE.IDATA(N-1))GO TO 8Ø
      ID1=-1
      ID3=ITIME+N*INT
      GO TO 8Ø
62    IF(IDATA(N,1).LE.IDATA(N-1,1))GO TO 66
63    ID1=1
      ID4=ID2-(IDATA(N-1,1)/2)
      ID2=IDATA(N,1)+ID2-ID4
```

```
      IF(ID4.LT.2Ø)GO TO 8Ø
      GO TO 75
66    IF(IDATA(N,1).GT.1)GO TO 76
      ID1=Ø
      IF(ID2.LT.2Ø)GO TO 8Ø
      ID4=ID2
75    ID4=ID4*INT
      WRITE(1,1ØØ2)ID4,ID3
1ØØ2  FORMAT(1H,218)
1ØØ3  FORMAT(8H PK AR T)
      GO TO 8Ø
76    ID=ID2+IDATA(N,1)
8Ø    CONTINUE
82    ITIME=ITIME+INT*NPOINT
      IF(NPOINT.EQ.72Ø)GO TO 1Ø5
      CALL TAPE(ISIG.INTA)
      IF(ISIG.EQ.1)GO TO 8
85    CALL ACSW(ISIG)
      IF(ISIG.EQ.Ø)GO TO 85
      GO TO 5
9Ø    ID1LAS=Ø
      GO TO 26
95    DO 1ØØ N=1,NPOINT
      IDATA(N,1)=IDATA(N,2)
1ØØ   CONTINUE
      NPOINT=NPOINT+3
      GO TO 22
1Ø5   READ(2,1ØØ4)NBAK
1ØØ4  FORMAT(I3)
```

```
      ITIME=ITIME-INT*NBAK
      DO 1Ø8 N=1,NBAK
      NSUB=NPOINT+N-NBAK
      IDATA(N,1)=IDATA(NSUB,2)
1Ø8   CONTINUE
      NPOINT=NBAK
      GO TO 15
1ØØ1  FORMAT(1H, I6)
11Ø   DO 12Ø N=3,NPOINT
      IDATA(N,1)=(IDATA(N-2,2)+IDATA(N-1,2)+IDATA(N,2)+
      IDATA(N+1,2)+IDATA(N+2,2))/5
12Ø   CONTINUE
      NPOINT=NPOINT+3
      GO TO 22
      END
```