The Application of Gas Liquid Chromatography to the Simultaneous Analysis of Sugars and Sugar Phosphates in Biological Samples.

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ABSTRACT

A study was undertaken to determine the applicability of gas liquid chromatography to the simultaneous analysis of sugars and sugar phosphates from biological samples. A new method of silylation involving dimethylsulfoxide, hexamethyldisilazane, trimethylchlorosilane and cyclohexane (1:0.2:0.1:1) which rapidly silylated sugars and sugar phosphates was developed. Subsequent chromatography on a 5% SE-52 column gave good resolution of the sugar and sugar phosphate samples. Sugar phosphates decomposed during chromatography and were lost at the $7 \times 10^{-3} \mu\text{mole}$ level. Acidic ethanol extraction of yeast samples revealed background contamination from the yeast sample, the culture medium and the silylation reagents which would further limit the level of detection obtainable with the glc for sugars in biological samples to the $3 \times 10^{-4} \mu\text{mole}$ level.
ACKNOWLEDGEMENTS

I would like to thank Dr. A.J.S. Ball and Dr. R. Hiatt for their assistance and guidance throughout this work.

I would also like to thank other members of the staff, and students, for their interest and concern in my thesis work.
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CHAPTER I
INTRODUCTION

This thesis describes an attempt to develop a new method of simultaneously analysing sugars and sugar phosphates rapidly and easily for the benefit of other workers studying sugar metabolism with special reference to yeast*. The usual methods of sugar analysis such as paper or column chromatography or enzymatic analysis possess deficiencies and drawbacks which limit their practicality (see Literature Review below).

Gas-liquid chromatography (glc), however, appears to be promising in that it (theoretically) has the sensitivity of enzymatic analysis, the advantages of a chromatographic technique and a speed and versatility all its own. Since metabolism studies would probably involve a large number of rapidly taken samples, the procedure for preparing volatile derivatives suitable for glc work should be a fast, easy and preferably one step procedure. Silylation derivatisation procedures combined with glc offer all these advantages. Ideal glc analysis conditions would have to be discovered and the lower limits of detection investigated as the quantity of material under study would be small. Problems associated with adapting this method to biological samples would have to be discovered and investigated.

In this investigation, the literature is of limited help. The glc of simple sugars is a standard procedure1 but there are only five papers

* This work was carried out in conjunction with Dr. A. Ball's studies into the glucosamine resistance of yeast cells.
in the literature on the glc of sugar phosphates. As each of the latter papers outlines a different experimental method, there are too few papers for a consistent pattern of success to appear. In fact, Birch notes that "the published work (on the glc of sugar phosphates) so far seems to be curiously inexplicit with regard to experimental detail". Alternately, Holligan notes that no systematic investigation of the limits of detection possible with the glc for simple sugars (or sugar phosphates) in biological material has been undertaken.

There are then, deficiencies in the literature which make an investigation of the simultaneous glc of sugars and sugar phosphates in general and from biological material in particular, a worthwhile undertaking.
Historical Perspective

The earliest tests for sugars were adapted from classical chemical procedures such as functional group analysis. Fehling's solution (1848) and Tollen's test (1892) were tests for aldehyde groups in general rather than for sugars in particular. Such tests have led to the modern distinction between reducing and non-reducing sugars.

In 1929, Dische outlined an osazone derivatisation procedure which provided the first systematic analysis of sugars. The bases of identification used were rate of reaction in forming the derivative, crystal shape, melting point, and polarimetry work on the derivative. The original sugar was not recoverable.

Fleury and Lange perfected the periodate oxidation technique discovered by Malaprade and developed the first analytic technique stereospecific for polyhydroxyl compounds, i.e., sugars. The sample is oxidized completely using periodate ion and the time of formation of formic acid and formaldehyde is noted over a period of 48 hours. This indicates the number and orientation of hydroxyl groups and hence the sugar.

In the 1940's, the colour reactions for sugars were developed. These tests were based on the formation of furfural from sugars in highly acidic solution and subsequent reaction with phenols to produce strongly coloured products.
Paper chromatography developed in 1944\textsuperscript{14}, enzymatic analysis (1948)\textsuperscript{15} and gas-liquid chromatography (1958)\textsuperscript{16} of sugars form the basis of the modern methods of sugar analysis to be discussed below. Each represents improvements over earlier methods and has added great impetus to the field of sugar analysis.

**Paper Chromatography**

Several good review articles on the theory and practice of paper chromatography exist\textsuperscript{17-20} although the technique has not changed substantially since the first published report\textsuperscript{14}; Benson\textsuperscript{21} reviews the paper chromatography of sugar phosphates since it was first done in 1946\textsuperscript{22}.

In paper chromatography, the sample is spotted near an edge of a sheet of filter paper and an organic solvent is allowed to flow past the sample spot. The sample moves with the solvent depending on its solubility in that solvent and this is reflected in the sample's $R_f$ value, i.e., the ratio of the distance the sample has moved with respect to the distance the solvent front has moved. Ratio values can also be defined with respect to an internal standard\textsuperscript{23}.

To overcome low $R_f$ values in solvent systems suitable for sugars, the solvent is allowed to flow past the far edge of the paper in the descending mode. Similar results can be achieved by repeated chromatogramming when ascending paper chromatography is used. In two dimensional chromatography, the sample is applied near one corner of the sheet of paper and then two developments are carried out, the second at $90^\circ$ to the first. The latter affords better resolution than the former, one dimensional methods. In any of these methods, 24 to 36 hours is required for complete development.
Detection of sugars by using methods from classical chemistry described above allows less than 0.01 μmoles of sugar to be detected\textsuperscript{24}. Quantification of the sample by comparing spot sizes with standard samples\textsuperscript{25} or elution for spectrophotometric work is tedious and prone to cumulative errors\textsuperscript{18}.

Solvents suitable for one class of sugars, e.g., sugar acids, will give elongated or multiple spots for other classes of sugars, such as sugar amines\textsuperscript{19} or sugar phosphates\textsuperscript{21} making the simultaneous analysis of different types of sugars by paper chromatography difficult if not impractical.

The chromatography of sugar phosphates requires the removal of ions from both the original samples and from the chromatography paper\textsuperscript{21} as ions can affect the sugar phosphate $R_f$ values. The $R_f$ values of sugar phosphates are also affected by other sugar phosphates in the sample, the degree of hydration of the paper, impurities in the solvent and the distance the solvent front has moved, making the determination of absolute $R_f$ values for sugar phosphates impossible. Lastly, sugar phosphate samples smaller than 0.01 μmoles will be lost due to irreversible binding at the origin and this limits sensitivity\textsuperscript{21}.

The problems associated with paper chromatography in general such as long analysis times and difficulty of quantification, combined with the method's inability to adequately separate sugars and sugar phosphates simultaneously makes the investigation of other means of analysis desirable.
Zone Electrophoresis

In zone electrophoresis, sugars treated with a borate buffer travel across a strip of wet paper under the influence of an applied voltage\textsuperscript{26,27}. The higher the voltage, the faster the development and the better the resolution. This is limited only by heating problems and general safety precautions associated with high voltages. Electrophoresis is faster than paper chromatography, a typical analysis taking only two hours.

The solvent is water and the important variable affecting the mobility of the sample is pH. Thus the system is easily changed to effect resolution of a wide range of compounds, but rarely simultaneously.

For consistent results, the experimental parameters, voltage, temperature and pH must be strictly controlled. A suitable internal standard, preferably coloured, is always required.

Apart from the more complex equipment required for development of the sample in zone electrophoresis, detection and quantification of the sample spots is the same as for paper chromatography and suffers from the same disadvantages.

Column Chromatography

Thomas et al.\textsuperscript{28} in 1974 reported the separation of sugar phosphates utilizing an automated column chromatography system which was a considerable improvement over other more laborious column chromatography systems, e.g., Benson\textsuperscript{21}. A column of BioRad-AG ion exchange resin containing the sugar phosphate sample (the column neither retains nor separates simple sugars) was maintained at 70° and sequentially eluted with three different buffers. Detection was by a photo-activated orcinol reaction at 105°. Recycle time was four hours.
With this system, Thomas et al., claim a sensitivity of better than one nanomole which is comparable to enzymatic analysis (below). However, the complexity of the system requiring proportioning pumps, two temperature controls, a UV lamp and a spectrophotometer with a flow through sample cell, all for the analysis of sugar phosphates only, does not make itself readily accessible to most biological laboratories.

**Enzymatic Analysis**

The modern method of enzymatic analysis developed by Otto Warburg in 1948 is generally based on the absorption of light by the coenzyme NADH at 340 nm. This compound is created from NAD$^+$ in enzymatic reactions linked to the compound (substrate) being analysed. This technique is capable of detecting as little a $3 \times 10^{-3}$ µmoles of substrate.

If NADH is exposed to the 365 nm line of mercury, it will fluoresce at 460 nm extending the sensitivity of enzymatic analysis by two or three orders of magnitude. This fluorometric method is however more difficult, requiring a sensitive fluorimeter with electronic modifications and careful reagent preparation.

The well-known specificity of enzymes allows a sugar to be analysed in the presence of other sugars and compounds normally present in acid-extracted biological samples, (the acid, usually perchloric, having been neutralized beforehand). Usually, a suitable enzyme or series of enzymes can be found to react with the substrate and cause the transfer of hydrogens to NAD$^+$ . For glucose-6-phosphate (G-6-P), a suitable enzyme is glucose-6-phosphate dehydrogenase (G-6-PDH). The analysis is fast (3 minutes) and the total change in absorbance at 340 nm is the parameter
measured. Fructose-6-phosphate (F-6-P) is analysed in the same sample by subsequently converting it to G-6-P by adding phosphoglucone isomerase (PGI). This is reacted upon by the G-6-PDH and this secondary change in absorbance is proportional to the F-6-P concentration.

One disadvantage of the method is the number of solutions which must be mixed together for each analysis. This makes the analysis of even a reasonable number of substrates a lengthy and complex task. Ultimately though, the method's great specificity is its greatest disadvantage when viewed as a general analysis method. The method cannot alert you to the presence of compounds that you are not specifically looking for and requires at least one enzyme per compound under study. Enzymes are often of questionable stability and might have to be isolated by the investigator as not all enzymes are commercially available. Despite these deficiencies, enzymatic analysis is the most sensitive and reliable method to date which can be used to analyse samples for sugars and sugar phosphates.

**Gas-Liquid Chromatography**

A book on the use of the gas-liquid chromatograph (glc) in biology exists as well as review articles on sugar sample preparation for glc analysis. The Holligan and Birch papers review the glc of sugars in plant material and foods respectively. Dutton, in two extensive but not exhaustive review articles lists 1,163 references relevant to the glc of simple sugars since the technique was first used in 1958.

In the glc, a sample solution of 10 µl or less is introduced into the "solvent" gas stream via a syringe through a septum stoppered inlet. The inlet area is called the injection port and is usually heated to
quickly vaporize the sample (see fig. 1). The gas stream, usually helium or nitrogen, carries the vaporized sample through a column packed with a solid support which has been coated with the stationary liquid phase. This column, usually 6' x $\frac{1}{4}'$, is in an oven compartment and is heated as necessary. The sample is partitioned between the stationary liquid phase and the gas stream and this determines its retention time, (the time taken for a particular compound to pass through the column) which is a constant for specified analysis conditions. If the liquid phase has been chosen correctly, the sample peak will resemble a gaussian curve when recorded.

Glc detectors tend to be universal, giving an output signal for any type of compound and are usually very sensitive. The thermal conductivity (or hot filament) detector measures the change in heat conductivity in the sample stream relative to a reference gas stream due to the presence of volatile compounds. This type of detector can detect as little as $10^{-3}$ μmoles of material$^{35}$. Flame ionization detectors are the preferred detectors for biological work. They are universal for organic compounds because when organic molecules burn they produce ions which can conduct electricity and thus be detected. The flame ionization detector is capable of detecting as little as $10^{-9}$ μmoles of material$^{35}$ bettering enzymatic analysis in sensitivity.

The stationary liquid phase is the most important factor affecting sample resolution and it must be stable at the analysis temperature, possess low volatility, high viscosity and be inert to the class of compounds under study. The amount of liquid phase which normally vaporizes contributes a background level to the detector signal and
Figure 1: Schematic Representation of a Gas-Liquid Chromatograph

1 - carrier gas inlet
2 - gas flow rate control
3 - temperature controls
4 - septum stoppered sample inlet
5 - injection port
6 - chromatography column
7 - oven compartment
8 - detector
9 - detector output
10 - strip chart recorder
is referred to as column "bleed". Column bleed and hence performance become critical near the liquid phase's maximum operating temperature. Some liquid phases suitable for the glc of simple sugars are described in Table 1.

When a broad range of compounds of widely ranging volatilities is to be analysed, constant temperature (isothermal) conditions are rarely adequate because of the long retention times experienced with the low volatility compounds. In such a case, the column temperature can be programmed to increase at a constant rate from a low to a high temperature to provide an optimal temperature for separating each class of compound during the analysis. In this case a retention temperature is given for a compound rather than a retention time. Thus, given the proper column conditions, no analysis need take longer than one hour.

Quantification of the glc data is relatively straight forward because sample size is generally proportional to the peak area as it appears on the strip chart recorder connected to the detector output. Holligan\(^8\) in reviewing methods of glc quantification, noted that if sufficient care is taken to ensure constant experimental conditions, peak height measurements are as accurate as any other quantification method and prone to less error. In all cases, a standard curve is considered routine for glc work.

Because a gas possesses low viscosity, longer and narrower columns and faster analysis times are possible, effecting better resolution than obtained with other chromatographic techniques\(^41\). Use of a gas allows the analysis temperature to be widely varied and the sample can be recovered from the gas stream by simple condensation\(^36,42\).
The versatility of the glc apparatus allows rapid changes in the analysis conditions and easily interchangable columns with different liquid phases allows for further modification. The sophistication of readily available glc machines facilitates automation, such as temperature programming so that sample analysis and even quantification (with electronic integrators) can occur without operator intervention once the sample has been injected.

The major disadvantage in the glc technique is that the sample must be volatile. Most organic molecules, notably sugars, are not volatile in their natural state under any but the most extraordinary circumstances. It is with the preparation of volatile derivatives for sugars and sugar phosphates that workers have largely concerned themselves. The following presents the three types of derivatives suitable for the glc analysis of sugars: the alkyl, the alditol acetate and the trimethylsilyl (TMS) derivatives.

Methylation is the most common way of obtaining alkyl derivatives and was the first to be used. Methylation can be carried out in a number of ways but these procedures suffer from the time required to prepare the sample, the tendency to give multiple peaks due to incomplete methylation and low volatility of the derivatives which makes it hard to analyse sugars of molecular weight higher than disaccharides.

The major use for the glc analysis of methylated sugars is in the structure determination of polysaccharides but even here, the partially methylated sugars are also silylated to increase their volatility.

In the alditol acetate derivatisation procedure, the aldose sugar is reduced to its corresponding alditol using sodium borohydride
followed by acetylation. This derivative allows the highest resolution of any of the derivatives discussed partly because there is only one peak per aldose sugar; keto sugars give two peaks though. Unfortunately, the long and complex derivatisation procedure necessitates an internal standard and is difficult to work with for small or numerous samples. Alditol acetate derivatives are not very volatile allowing only monosaccharides to be analysed. Because of these shortcomings, alditol acetates are used to confirm a sample assignment from the silylation procedure (below) rather than as a primary analytic method.

The aldose sugars themselves are not acetylated because in practice, silylated aldoses afford better resolution. The reasons for this are discussed by Birch and Holligan.

The silylation procedure developed by Sweeley et al. for simple sugars is a one vial, 10 minute procedure. A dry sugar sample (10 mg) is dissolved in one ml of pyridine and 0.2 ml of hexamethyldisilazane (HMDS) (I) and 0.1 ml of trimethylchlorosilane (TMCS) (II) are added. The reaction is as in equation 1 and is quantitative requiring no internal standard. In some silylation reactions, dissolution of the sample in the silylation reagents is taken to be sufficient criteria for silylation to have occurred.

\[
\begin{align*}
((\text{CH}_3)_3\text{Si})_2\text{NH} & \quad \text{(I)} \\
(\text{CH}_3)_3\text{SiCl} & \quad \text{(II)}
\end{align*}
\]

Equation 1: \[\text{CH-OH} + \text{HMDS} + \text{TMCS} \xrightarrow{\text{PYR}} \text{CH-O-Si(CH}_3)_3 + \text{NH}_4\text{Cl}^{+}\]

Sweeley reported the glc of tetrasaccharides and even heptasaccharides have been chromatogrammed. The trimethylsilyl (TMS) group is susceptible to hydrolysis and requires anhydrous handling procedures.
although it allows easy recovery of the original sugar if necessary. Chromatography of the silylated sugar samples can be carried out on column packings ranging from the highly polar Carbowax 20M to the non-polar SE-30, Table 1.

In the latter silylation procedure, the sample is dissolved in 1.3 ml of solution, a relatively large sample volume which reduces the sensitivity possible with this method. Further, pyridine tends to tail badly on most liquid phases, obscuring chromatogram peaks. Attempts have been made by other workers to remove the pyridine by evaporation or extraction but these add steps to an otherwise simple derivatisation procedure.

Ellis investigated the use of other silylation solvents, dimethylformamide (DMF) and dimethylsulfoxide (DMSO). The silylated derivatives (including hexamethyldisiloxane, a side product from silylating water in the sample) are not soluble in either solvent and form a second (upper) liquid phase which has a small volume and high sample concentration. Formation of the upper phase takes 10 minutes in DMSO and 18 hours in DMF.

Wells et al. in 1964 reported their failure to directly silylate sugar phosphates using pyridine, HMDS and TMCS. They then chose to premethylate the phosphate group using diazomethane in methanol and then silylate the rest of the sugar phosphate molecule. However, Hashizume and Sasaki achieved total silylation of some sugar phosphates by refluxing the sample with pyridine, HMDS and TMCS. Eisenberg and Bolden achieved similar results by heating in a closed vial at 100°. None of these methods succeeded in forming derivatives of glucose-1-phosphate (G-1-P). Eisenberg and Bolden reported only a 50% recovery of G-6-P after glc work implying a need for an internal standard. Concentration
<table>
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<th>Structure</th>
<th>Maximum Operating Temperature (°C)</th>
<th>Molecular Weight</th>
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<tr>
<td>Carbowax 20M</td>
<td>Very polar</td>
<td>OH ((-\text{CH}_2\text{-})_n)</td>
<td>250</td>
<td>Highest of the Carbowax series.</td>
</tr>
<tr>
<td>SE-30</td>
<td>Non-polar</td>
<td>CH(_3) ((-\text{Si-O-})_n) (\text{CH}_3)</td>
<td>300</td>
<td>3,000,000</td>
</tr>
<tr>
<td>SE-52 (phenyl)</td>
<td>Intermediate</td>
<td>CH(_3) ((-\text{Si-O-})_n) (\text{Ph})</td>
<td>300</td>
<td>3,000,000</td>
</tr>
<tr>
<td>XE-60 (nitrile)</td>
<td>Intermediate</td>
<td>CH(_3) ((-\text{Si-O-})_n) (\text{CN})</td>
<td>275</td>
<td>high</td>
</tr>
<tr>
<td>OV-1</td>
<td>Non-polar</td>
<td>similar to SE-30</td>
<td>300</td>
<td>3,000,000+</td>
</tr>
<tr>
<td>OV-25</td>
<td>Intermediate</td>
<td>similar to SE-52</td>
<td>300</td>
<td>30,000</td>
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of the sample under strictly anhydrous condition failed to yield a stable sample and resulted in an "anomalous peak pattern".\(^5\)

Sherman et al. in 1971\(^6\) developed a room temperature silylation procedure for sugar phosphates (including G-1-P) using pyridine, TMCS and bis(trimethylsilyl)acetamide (BTSA) (III):

\[
\begin{align*}
\text{O-Si(CH}_3\text{)}_3 \\
\text{CH}_3\text{-C=N-Si(CH}_3\text{)}_3
\end{align*}
\]

(III)

BTSA had not been used previously for the routine silylation of simple sugars because it tends to give inconsistent results\(^4\). Horning et al. also used BTSA to silylate sugar phosphates\(^4\).

Sherman noted "spurious" peaks in his samples which grew with time to rival the sample peaks in size. The peaks were present in BTSA from various sources and were enhanced by the silylation procedure. He also noted that some new GLC columns required "priming"; i.e., repeated injections of silylated sugar phosphates before they would yield a consistent peak size. Alternately, some previously useful columns would gradually fail to yield a sample peak. The latter effect was attributed to the buildup of decomposition products on the column, notably phosphoric acid, which act as catalytic sites for decomposition of the sample.

Despite the difficult and imperfect silylation procedures for sugar phosphates, the above work indicates that the simultaneous analysis of sugars and sugar phosphates will be relatively easy as these silylated derivatives tend to have retention times which are proportional to their molecular weight\(^3\) with variations due to stereochemistry\(^7\). This is in sharp contrast to paper or column chromatographies which do not have
such clear-cut separation criteria. The results below outline the experiments which led to a simple one step silylation procedure for the simultaneous silylation of sugars and sugar phosphates. The experiments also indicate the usefulness and limitations of this technique for analysing biological samples using the glc method.
CHAPTER 3
MATERIALS AND METHODS

Chemical and Chromatography Supplies

The following standards were obtained from Sigma Chemicals, (St. Louis, Missouri) at 98% or better purity: N-acetyl glucosamine, (NAcGNH2); glucosamine-6-phosphate, (GNH2-6-P); glucose-6-phosphate, (G-6-P); glucose-1-phosphate, (G-1-P); fructose-6-phosphate, (F-6-P) and fructose-1,6-diphosphate, (FDP).

The following reagents were obtained from Pierce Chemicals (Rockford, Illinois) and are of silylation grade: bis(trimethylsilyl) acetamide, (BTSA); trimethylchlorosilane, (TMCS); hexamethyldisilazane, (HMDS); pyridine, (pyr); dimethylsulfoxide, (DMSO); dimethylformamide, (DMF); Silyl-8 and dimethylchlorosilane. The stationary liquid phases OV-1 and OV-25, a "Pressure-Lok" syringe (10 µl), "Tuf-Bond" teflon faced silicone rubber septa and 3.5 ml screw cap vials were also obtained from Pierce Chemicals.

Varian Aerograph (Walnut Creek, California) supplied the stationary liquid phases, SE-30, SE-52 and XE-60.

Chromatography Specialties (Brockville, Ontario) supplied the Carbowax 20M liquid stationary phase and the solid support, 80/100 Chromosorb W.

Difco Yeast Nitrogen Base (w/o amino acids) and Difco Bacto-Dextrose were purchased from the BDH Chemical Company (Toronto, Ontario).

All other chemicals were obtained as the highest possible grade available from BDH.
The gas chromatograph was a Hewlett-Packard 5700A temperature programmable digital gas chromatograph equipped with flame ionization detectors and outfitted for dual column operation. The detector signal from the gas chromatograph was recorded using a Fisher Recordall Series 5000 strip chart recorder (Fisher Scientific Company). The hydrogen and prepurified nitrogen carrier gas were from the Linde Corporation (supplied by Niagara Welders Limited, St. Catharines, Ontario).

Purification of Reagents

The silylation grade reagents and solvents were packed under nitrogen in septummed bottles and used as supplied by Pierce Chemicals.

The 95% ethanol was distilled once and diluted to an 80% concentration with distilled water.

The cyclohexane was purified by the following procedure as outlined in the Chemist's Companion. Cyclohexane was first washed with a 2:1 mixture of concentrated sulphuric and nitric acids, then washed three times with water and finally dried over calcium chloride. The cyclohexane was then distilled over calcium chloride and subsequently passed through a short column of acid washed activated alumina. The purity of the cyclohexane was monitored throughout using the glc.

Deactivation of the Solid Support

The chromatography solid support, 80/100 Chromosorb W was deactivated following the method reported by Leibrand and Dunham. The Chromosorb W (50 gm) was put into a flask fitted with a septum stopper and the flask flushed with dry nitrogen. 50 µl of dimethylchlorosilane was introduced and the mixture allowed to stand for two hours when 100 µl of methanol was added. After a further two hours, the mixture
was transferred to a sintered glass funnel and washed with isopropyl alcohol. The Chromosorb W was then dried under a stream of nitrogen and stored in a closed container.

This procedure was also used for the silylation of some glass wool.

**Preparation of the Glc Column**

Enough SE-52 silicone oil was weighed out to give a 5% coating onto the Chromosorb W. The SE-52 was dissolved in 150 ml of dichloromethane and the Chromosorb W added to this mixture. The solvent was removed under vacuum in a rotary evaporator and the powder left overnight to air dry in a shallow dish. Drying was continued until all trace of solvent had disappeared.\(^{35}\)

One end of the chromatography column was plugged with silylated glass wool and an aspirator was attached. The column packing was introduced with occasional tapping to assist powder flow. The other end of the column was then plugged with the silylated glass wood.

The column was conditioned by heating the glc at 300° for 1 to 3 hours with normal carrier gas flow. Silyl-8 was used according to the instructions supplied on the label to ensure proper conditioning.

**Silylation of Standards**

The following is a modification of the silylation system reported by Ellis\(^{50}\) and represents the final method developed in this work. This system was found to be suitable for the silylation of sugars, sugar phosphates and yeast extract samples (below). No special care is required when silylating standards except that the reagents need to be transferred by syringe and the silylated sample has to be protected from moisture by use of a rubber septum stopper.
Ten milligrams of the solid standard sample is weighed into a vial and one ml of DMSO and one ml of cyclohexane are added to the vial. The vial is capped with a septum stopper and 0.2 ml of HMDS and 0.1 ml of TMCS are added in that order. The vial is then shaken or, preferably, placed in a sonicating water bath until the sample dissolves. It is neither necessary nor recommended that attempts be made to dissolve the sample before the silylating reagents are added. Heating of the sample is to be avoided.

The sample is silylated upon dissolving and the silylated compound will be found in the upper (cyclohexane) phase. The sample is stable for up to one month if stored upright in the refrigerator (though some minor peaks may appear within two or three days). More cyclohexane can be added as needed to offset evaporation of the upper phase.

Gas-Liquid Chromatography of Samples

The most useful column was 6' x \frac{1}{4}" glass packed with 5% SE-52 on 80/100 mesh silanized Chromosorb W. The injection port temperature was 150° and the detector temperature was 300°. The column temperature was programmed from 140° to 290° at 4°/min. The carrier gas was prepurified nitrogen passed through a molecular sieve drying tube. The carrier gas flow was 60 ml/min as recommended by the manufacturer.

The paper speed on the strip chart recorder was 1/10"/min and the full scale sensitivity was 1 mv.

Preparation of Yeast Samples

The yeast strain used was a diploid of \textit{S. cerevisiae} (strain 411 from the collection of Dr. A. Ball, Brock University) grown routinely at 30° on a YPD slope (1% yeast extract, 2% peptone, 3% dextrose and 2% agar) and stored at 4°C.
The diploid yeast strain was introduced aseptically by loop inoculum into 50 ml of SD liquid medium (3% dextrose, 0.5% Difco Nitrogen Base w/o amino acids) in a 250 ml flask. The culture was allowed to grow at 30° with a shaking rate of 350 r.p.m. until the culture reached an optical density of 0.6 to 0.8 at 415 nm (approximately 14 hrs). Samples were harvested by passing 10 ml of the culture through a Millipore filter (25 mm, 0.45 μm) and as quickly as possible transferring the filter plus yeast into 3.5 ml of 80% ethanol acidified with 5 μl of 5N HCl. This was accomplished in 8 to 10 seconds. The vial was capped and shaken vigorously to remove the yeast from the filter. The filter was then removed and the sample placed in a sonicating water bath for ten minutes.

The sample extract was then spun down in a clinical centrifuge at maximum speed for one minute and 3.0 ml of the supernatant was transferred to a preweighed silylation vial. The sample was dried under vacuum to constant weight.

Silylation of Yeast Samples

The vial containing the dried ethanolic yeast extract was resealed with a teflon faced silicone rubber septum. All reagents were handled in either an all-glass syringe with replaceable stainless steel needles or a similar syringe with a teflon plunger. The syringe was rinsed at least once with the appropriate reagent before the reagent was transferred to the sample vial.

Into the sample vial was placed 1 ml of DMSO and 0.3 ml of cyclohexane. For a series of samples, enough of the silylating reagents were added to ensure complete silylation of the largest sample. For 2 to 4 mg samples, 50 μl of HMDS and 25 μl of TMCS added in that order was generally adequate. The samples were sonicated (not shaken) until
dissolved. The DMSO may be removed using a long needle syringe to facilitate sampling from the small, upper layer of cyclohexane.

The samples are stable at room temperature for up to 24 hours but spurious peaks will gradually appear after 24 hours. A millipore and/or reagent blank is useful in this regard. The samples cannot be stored under refrigeration.
CHAPTER 4
RESULTS

This study was undertaken to develop a technique which would allow the easy monitoring of short lived metabolites, specifically, sugar and sugar phosphates in yeast. Enzymatic analysis (done previously by A. J. S. Ball\textsuperscript{54,55}) although having the sensitivity required, was considered to have disadvantages severe enough to warrant a look at other techniques (see also Literature Review).

Some form of chromatographic analysis was indicated by the literature as being most useful and preliminary work was begun using thin-layer chromatography (with principles and techniques adapted from paper chromatography). The technique in our hands did not appear capable of resolving even a reasonable number of sugars nor did any single solvent system appear useful for a wide range of sugars and sugar derivatives.

Paper chromatography was not tried because of its limitations as seen in the literature (see Literature Review).

Gel electrophoresis was tried briefly based on the success of Sjodins and Vestermark\textsuperscript{56} with electrophoresis. It was found not to separate glucose and glucosamine. Further, the usual sugar colour developers, adapted from paper chromatography, tended to destroy the gel.

Gas-liquid chromatography (glc) has potentially the same sensitivity as enzymatic analysis, combined with speed, accuracy and simplicity of operation. Thus, glc would be the preferred method if technical problems could be overcome.
The rest of the results section is concerned with the attempts to overcome the problems encountered in applying the glc technique to the separation and identification of sugar metabolites in yeast.

The results lend themselves to division into three major sections, Silylation, Chromatography and Yeast Studies. Each section is presented chronologically except where clarity is served by presenting points out of chronological order. The figures referred to are meant to be representative of the point under discussion and may be discussed further in subsequent sections because some problems necessarily occur simultaneously. For example, silylation reactions often give visible indications of reaction (solution being a necessary condition) but the proof of silylation is the appearance of glc peaks on a suitable column. As such columns must often be chosen by trial and error, the explanation for the absence of peaks in any one instance is often indeterminate. We have therefore decided to subordinate the discussion of column changes in the silylation section and defer a detailed reporting of such changes until the chromatography section.

Silylation

I. A convenient starting point in the work was the method reported in the literature by Sweely et al.45 Pyridine, hexamethyldisilazane and trimethylchlorosilane (pyr/HMDS/TMCS proportions being 1:0.2:0.1) was reacted with 5-10 mg of solid samples of simple sugars (glucose and galactose) by sequentially injecting the reagents (pyr/HMDS/TMCS) into a closed septummed vial in that order. Silylation occurred as indicated by the formation of a fluffy ammonium chloride precipitate and the
appearance of peaks in the glc on SE-30. Isothermal conditions (160° to 180°) were tried first but during the chromatogramming of the samples, pyridine "tailed" badly. Temperature programming of the column oven was necessary to gain reasonable resolution of the sample peaks from the solvent (pyridine) and also to obtain sharp sample peaks. Attempts to use pyr/HMDS/TMCS on glucosamine (GNH₂) and glucose-6-phosphate (G-6-P) yielded no visible reaction.

Pyridine tailing was considered to be a major problem and one solution to this was to try other silylation solvents (as outlined by Pierce). II. Other solvents available for silylation were acetonitrile, dimethylsulfoxide (DMSO), tetrahydrofuran (THF) and dimethylformamide (DMF). Each solvent was used with the usual silylating reagents (HMDS and TMCS) and glucose. Acetonitrile and DMSO yielded only broad peaks rather than the sharp one characteristic of glucose.

An upper layer was not expected with DMSO (IIIb below) and was not seen. DMF and THF both permitted the silylation of glucose (faster than with pyridine as solvent) and yielded a normal sharp glucose peak in the glc. However, the THF system also yielded large impurity peaks (also present in the original solvent) and so DMF was chosen as solvent in the subsequent tests on a range of compounds. [Samples were discarded after 12 hours as recommended by Pierce. As no separation of phases took place within 12 hours (see section IIIc(ii) below) the second phase was never observed (Ellis)].

DMF as solvent allowed the silylation of glucose and galactose to give one principal peak for each in the glc and silylated GNH₂ to give
three peaks, rather than one as expected. Individually silylated glucose, galactose and GNH₂ samples were combined for the chromatogram in Fig. 2 (see also Chromatography below). In general though, DMF as solvent proved disappointing as it did not induce silylation of either N-acetylglicosamine (Nac-GNH₂), sugar phosphates, representative amino acids or Krebs cycle intermediates.

III. A return was made to the pyridine solvent system and variation of reaction conditions and/or silylation reagents were tried in attempts to silylate sugar phosphates and GNH₂ properly.

(a) The first attempts were with the usual reagents (pyridine, HMDS and TMCS) plus heating. The reaction mixture was heated by immersing the vials in boiling water. Neither GNH₂ nor G-6-P showed any tendency to dissolve or silylate under these reaction conditions.

The next system tried was basically that of Sherman⁶ (see Introduction) where bis(trimethylsilyl) acetamide (BTSA) is used in place of HMDS. With these reagents glucose yielded four peaks immediately after solution was complete. This changed to one peak after three hours. GNH₂ was slow to react and yielded three peaks not unlike those obtained with DMF as solvent (Fig. 2) except that the peaks were of almost equal height and showed little tendency to change with time. G-6-P showed no tendency to dissolve or react with this system (pyr/BTSA/TMCS).

Another attempt to replace HMDS was made but this time triethylamine was used to replace HMDS as a base to promote the silylation with TMCS (which yields HCl as a side product of silylation). Intense brown discolouration of the silylation mixture occurred: glucose was silylated and GNH₂ was not. This system was abandoned because of the intense discolouration produced during the reaction.
Figure 2: Simple Sugars on Carbowax 20M

Injector Temp.: 200°   Detector Temp.: 300°
Column: 5% Carbowax on 80/100 Chromosorb W
       6' x 1/4" glass
Column Temp.: 100° to 250° at 4°/min
Attenuation: 10 x 128*
Samples:  5.8 mg galactose
          10.3 mg glucose
          7.3 mg glucosamine
Each was silylated separately in 0.5 ml DMF 0.2 ml HMDS and
0.1 ml TMCS. 3 μl samples were combined in the syringe to
obtain this chromatogram

Peaks:  1 solvent
        2 galactose
        3 glucose
        4 glucosamine (triplet)
        5 unknown

* The sensitivity of the glc is inversely proportional to the
numerical value of the attenuation; e.g., high attenuation means
small detector output and reduced sensitivity.
A final attempt was made by changing the solvent from pyridine to DMF and the silylating reagent to BTSA. G-6-P dissolved in the reaction mixture, but the multiple peaks (Fig. 3) which arose indicated that no useful silylation was taking place.

Experiments with the Sweeley\textsuperscript{45} system of silylation (above sections I and II) were discontinued when the Ellis\textsuperscript{50} system was discovered in the literature.

(b) The Ellis system utilized DMSO as solvent and HNDS and TMCS as silylating reagents (DMSO/HMDS/TMCS in the ratio 1.0:0.2:0.1). The system is characterized by a partitioning into two phases, the upper phase (which takes ten minutes to form) consists of the products of silylation and the lower layer consists of the original DMSO.

The system was tried on glucose and galactose and these were silylated readily and appeared in the upper phase as expected. This silylation mixture also silylated GNH\textsubscript{2} as one peak and was in this respect an improvement over all previous silylation reagent mixtures (above).

G-6-P also showed a tendency to dissolve in this silylation mixture (DMSO/HMDS/TMCS) faster than it did in DMSO alone but no peaks were observed in the glc. This failure to find a G-6-P peak was attributed to the silylation mixture not being powerful enough. The chromatography column (Carbowax 20M) was not suspected at this time because it was the best column tried so far (see Fig. 2 and Chromatography below).
Figure 3: G-6-P Silylation with BTSA.

Injector Temp.: 200°  Detector Temp.: 300°
Column: 5% carbowax 20M
       6' x 1/4" glass
Column Temp.: 110° to 250° at 4°/min.
Attenuation: 10 x 128

Sample: 2.6 mg of G-6-P reacted with 0.5 ml DMF, 0.3 ml BTSA and 0.1 ml TMCS.
Peaks: 1 solvent
       2 Unknown
       3 assumed to be G-6-P anomerization products.
(c) An attempt was made to increase the silylating power of the silylation (in DMSO) by precipitating out the chloride ion as silver chloride; (the chloride ion must be lost by TMCS before silylation is complete). The reactions which were thought to occur are given below:

\[(i-iii)\]

1. \[(\text{CH}_3\text{)}_3\text{Si}-\text{Cl} \iff (\text{CH}_3\text{)}_3\text{Si}^+ + \text{Cl}^-\]
2. \[\text{Cl}^- + \text{Ag}^+ \rightarrow \text{AgCl} \downarrow\]
3. \[(\text{CH}_3\text{)}_3\text{Si}^+ + (\text{PO}_3\text{)}(\text{sugar}) \rightarrow (\text{CH}_3\text{)}_3\text{Si}-\text{PO}_3\text{-(sugar)}\]

G-6-P was successfully silylated and Fig. 4 shows the chromatograms of the G-6-P/DMSO/TMCS sample on Carbowax 20M both before and after the addition of silver nitrate. Unfortunately, none of the samples survived for a second injection (whether it was a simple sugar or a sugar phosphate). This would make peak verification by co-chromatography difficult if not impossible. This, plus a vigorous evolution of gas bubbles while the sample was awaiting chromatogramming and the highly exothermic nature of the initial reaction caused us to attempt further modifications.

(d) The very high temperatures at which the G-6-P peak was coming out was causing excessive bleeding of the Carbowax 20M column. SE-52 was chosen to replace Carbowax 20M at this time. (For details see Chromatography below.)

Two modifications of the reaction mixture were tried: (i) replace silver nitrate with silver carbonate (to remove the potentially reactive nitrate ion from the system), and (ii) replace the silver nitrate with lead nitrate (to try another precipitating ion). Both replacement salts showed similar silylating ability when compared to silver nitrate but also resulted in the loss of the sample before another injection.
Figure 4: Silylation of G-6-P with DMSO/TMCS/Ag⁺

Injector Temp.: 200°  Detector Temp.: 300°
Column: 5% Carbowax 20M
6' x 1/4" glass
Attenuation: 10 x 28
Sample: 10 mg G-6-P dissolved in 1 ml DMSO and 0.3 ml TMCS added. To the above was added 10% AgNO₃ (in DMSO) until no more AgCl formed (0.4-0.5 ml).

Chromatogram A: sample before AgNO₃ was added.

B: sample after AgNO₃ was added.
Peaks: 1 solvent
2 G-6-P (silylated)
could be made. The other disadvantages previously described (exothermic etc.) were also present.

We could see no way to overcome the problems inherent in this DMSO/TMCS/Ag⁺ system and therefore we discontinued these studies.

(e) (i) Because I was now working with a new type of column (SE-52), I decided to return to the previous silylation mixture (DMSO/HMDS/TMCS) and look again at the ready solution of G-6-P in the reaction mixture (solution often being indicative of silylation, see Literature Review). This time, a sample of G-6-P was heated in the reaction mixture overnight at 55°. This procedure yielded a large peak in the glc. Similar work with glucose-1-phosphate (G-1-P), fructose-6-phosphate (F-6-P) and glucosamine-6-phosphate (GNH₂-6-P) yielded sharp high temperature peaks. G-1-P and to a lesser extent GNH₂-6-P showed signs of decomposition, as indicated by a broad low temperature peak (Fig. 5). FDP did not appear except as a broad peak under F-6-P. Fig. 6 shows the benefit of heating on the G-6-P silylation reaction. An extended heating experiment revealed that G-6-P and GNH₂-6-P were partially degraded to α-glucose and α-GNH₂ respectively. G-1-P did not yield glucose as a decomposition product.

During the chromatogramming of GNH₂-6-P it was seen that the GNH₂-6-P peak height and shape varied from one injection to another for the same sample. It was discovered that the GNH₂-6-P was decomposing in the injection port. Reduction of the injection port temperature from 200° to 150° allowed reproducible GNH₂-6-P peaks in the glc.

During the heating of the sample, the upper phase of the reaction mixture evaporated through the septum. Steps were taken to counteract this tendency.
Figure 5: Decomposition of GNH$_2$-6-P and G-1-P.

Injector Temp.: 200°  Detector: 300°
Column: 10% SE-52 on 80/100 Chromosorb W.
6' x 1/4" glass
Column Temp.: 130° to 250° at 8°/min, then held at 250° isothermal
Attenuation: 10 x 128

Chromatogram A: 10.4 mg GNH$_2$-6-P after 12 hours at 55°C in DMSO/HMDS/TMCS (1.0:0.2:0.1) 5 µl injection
Peaks: 1 Solvent
2 GNH$_2$-6-P decomposition peak
3 GNH$_2$-6-P
4 unknown

B: Same sample as above with 26 hours of heating at 55°. 5 µl injection.
Note the increased size of the lower temperature (decomposition) peak.

C: 10.2 mg G-1-P in DMSO/HMDS/TMCS (1.0:0.2:0.1) heated for 12 hrs at 55°C. 6 µl injection
Peaks: 1 solvent
2 G-1-P decomposition peak
3 G-1-P
4 unknown

D: Same G-1-P sample after 24 hours heating.
Figure 6: Effect of Heating on G-6-P

Injector Temp.: 200°  Detector Temp.: 300°
Column: 10% SE-52
  6' x 1/4" glass
Column Temp.: 130° to 250° at 8°/min. then held isothermal at 250°
Attenuation: 10 x 128

Sample: 10.9 mg G-6-P treated with DMSO/HMDS/TMCS (1.0:0.2:0.1)

Chromatorgam A: sample heated at 55° for three hours (until sample dissolved). 3 µl injection.
Peaks: 1 solvent
2 G-6-P

B: the same sample heated at 55° for 14 hrs.
3 µl injection.
Peaks: 1 solvent
2 G-6-P
3 unknown

Note the smoothing of the front of the sample peak.
(e) (ii) To offset evaporation of the upper phase and thus protect the sample, (also to allow easier sampling) a solvent immiscible with DMSO was sought with which to augment the upper phase.

Solvents useful for extractions of a polar liquid (usually water) are chloroform, benzene, ether and cyclohexane. Only the last was immiscible with DMSO and was subsequently tried.

After the use of cyclohexane in the reaction mixture began, the silylation results became contradictory to the results obtained when cyclohexane was not used. Heating of the reaction mixture did not assist the reaction but caused excessive decomposition. Results obtained for GNH$_2$-6-P now showed multiple peaks after only 5 hours of heating (compared to 12 hours in the previous mixture (IIIe(i))).

This system (DMSO/HMDS/TMCS/cyclohexane) was now tried at room temperature. Figures 7 and 8 show the results obtained for sugars and sugar phosphates respectively. Individually silylated samples were combined to obtain these chromatograms. Chromatogramming of the samples immediately upon solution of the sample showed that the silylation reaction was essentially complete and thus solution of the sample was taken as sufficient criterion for silylation. The silylated derivatives were always found in the (upper) cyclohexane layer. Table II gives relevant glc retention temperature data and comments for various yeast metabolites.

The value of cyclohexane in promoting the silylation of G-6-P was confirmed later with dimethylformamide (DMF) as solvent (DMF is the other usable solvent for the Ellis system and was used to verify consistency in results). Silylation for G-6-P in DMF occurs in 18 hours as indicated by the formation of the second phase in this solvent.
Figure 7: Chromatography of Sugar Standards.

Injector Temp.: 150°   Detector: 300°
Column: 5% SE-52
       6' x 1/4" glass
Column Temp.: 110° to 280° at 2°/min
Attenuation: 10 x 64

Sample: Sugars silylated separately and combined in one vial for this run. Sample volume 10 µl.

Peaks: 1 solvent
       2 fructose (mannose) doublet
       3 α-galactose
       4 α-glucose
       5 α-glucosamine
       6 sorbitol
       7 β-glucose
       8 N-acetylg glucosamine
       9 unknown
       10 sucrose (maltose)
Figure 8: Chromatography of Sugar Phosphate Standards

Injector Temp.: 200°  Detector: 300°
Column: 5% SE-52 on 80/100 Chromosorb W
         7' x 1/8" copper
Column Temp.: 160° to 280° at 2°/min

Sample Sugar phosphates were silylated separately and combined for this chromatogram. (Excess G-1-P was added in order to reveal all of the G-1-P peaks).

Peaks: 1 solvent
       2 G-1-P decomposition caused by the copper column
       3 F-6-P
       4 G-1-P and G-6-P
       5 GNH₂-6-P
       6 unknown

The high retention temperatures are a result of restricted carrier gas flow.
**Table II: Chromatography of Standards**

Silylation Conditions: approximately 10 mg of solid sample was reacted in a septum stoppered vial. DMSO (1 ml), HMDS (0.2 ml), TMCS (0.1 ml) and cyclohexane (1 ml) were added in that order. The vial was then sonicated at room temperature until the sample dissolved.

Chromatography Conditions: Injection Port Temp.: 150°; Detector Temp.: 300°
Column: 5% SE-52 on silanized 80/100 Chromosorb W, 6'x1/4" glass.
Column Temp.: 140° to 290° at 4°/min.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Retention Temperature (°C)</th>
<th>Time taken to silylate (min.)</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amino acids</td>
<td>140</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Glycerol</td>
<td>140</td>
<td>1</td>
<td>Not resolvable from the solvent under these conditions.</td>
</tr>
<tr>
<td>Succinic acid</td>
<td>166</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Fructose, Mannose</td>
<td>182</td>
<td>1</td>
<td>Doublet</td>
</tr>
<tr>
<td>α-Galactose</td>
<td>188</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>α-Glucose</td>
<td>191</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>α-Glucosamine</td>
<td>194</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Sorbitol</td>
<td>196</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>β-Glucose</td>
<td>201</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>N-acetyl glucosamine</td>
<td>213</td>
<td>5</td>
<td>Close doublet</td>
</tr>
<tr>
<td>Fructose-6-phosphate</td>
<td>219</td>
<td>10</td>
<td>Decomposes to appear as an asymmetric peak under F-6-P.</td>
</tr>
<tr>
<td>Fructose-1-6-diphosphate</td>
<td>219</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Glucose-1-phosphate</td>
<td>229</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Glucose-6-phosphate</td>
<td>229</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Glucosamine-6-phosphate</td>
<td>245</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Sucrose, Maltose</td>
<td>260</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>
With the addition of cyclohexane to DMF, an upper phase was created immediately. G-6-P was then found to be completely silylated upon solution, which occurs in one hour. By comparison, solution was observed after 10 minutes in the DMSO (as solvent) system.

DMF and cyclohexane were found to be miscible with mild heating in the presence of the silylating reagents and occasionally this leads to the loss of the two phase system. This observation together with the slower reaction time (see above) makes DMSO the preferred solvent.

(f) A precipitate of ammonium chloride formed when pyridine was solvent and the ideal ratio of HMDS to TMCS was found to be 2:1. This ratio optimized the precipitation of Cl⁻ as NH₄Cl during the reaction (sections I and IIIc).

With DMSO as solvent, no precipitate forms and the ideal reaction conditions therefore may be different. The following experiments were designed to explore this question.

The usable proportions of HMDS to TMCS were investigated by using a series of vials containing G-6-P or GNH₂-6-P (10 mg per vial) to which were added 1 ml DMSO, 1 ml of cyclohexane and 0.3 ml of HMDS (to ensure a sufficient minimum quantity of a silylating reagent).

A different quantity of TMCS was subsequently added to each vial and the samples shaken until the sugar phosphate dissolved. Chromatography of the samples showed that the HMDS:TNCS ratio could be varied from 4:1 to 1:1 with little effect on the silylation reaction.
though the last concentration caused decomposition in the DMSO phase. Putting TMCS into the vial first also caused decomposition in the DMSO phase. The procedure finally adopted was to add the HMDS first and then add TMCS. The HMDS:TMCS ratio was maintained at 2:1.

Throughout the work, cyclohexane was the major component of the upper phase. This was especially convenient for smaller samples where proportionally less silylation reagents are used. This larger upper layer made it easier to extract glc samples from these reaction vials. Table III shows the best silylation conditions for the DMSO/HMDS/TMCS/cyclohexane system. (See also Yeast Studies below.)

(g) Silylation of samples up until this time occurred in individual vials, one compound per vial. Silylated samples were then mixed together to obtain the mixed sample chromatograms seen in Figs. 2, 7 and 8. In preparation for the calibration curves (below), solid glucose, NAc-GNH₂, F-6-P and GNH₂-6-P were weighed out into one vial and silylated together (with a proportionate increase in silylating reagents). This was done twice and all except GNH₂-6-P silylated reproducibly. GNH₂-6-P did not silylate at all. In one of these samples an effort was made, using sonication and heat, to get all of the sugars and sugar phosphates into solution before adding HMDS and TMCS. In this sample the sonication and preheating almost completely degraded GNH₂-6-P to GNH₂ (which appeared as a single peak) while the other compounds were unaffected. An earlier attempt consisting of only G-6-P, F-6-P and GNH₂-6-P (without heating) also failed to yield a large unique peak for GNH₂-6-P; neither was any GNH₂ found in this sample.

As the primary objective at this time was to do calibration curves (below) this disappearance was not investigated further.
Table III
Optimal Silylating Conditions

Vial: 3.5 ml with teflon faced silicone rubber septum.
Sample size: 10 mg for standards.

Reagent Volumes:

- Dimethylsulfoxide (DMSO) silylation grade: 1.0 ml
- Hemanethyldisilazane (HMDS): 0.2 ml
- Trimethylchlororosilane (TMCS): 0.1 ml
- Cyclohexane: 1.0 ml

Reagents added in this order

Procedure:
The vial is sonicated in a water bath a 22°-25° until the sample is dissolved. Excessive heating should be avoided.

The volume of DMSO is always kept equal to or greater than 1 ml. The silylating reagents can be altered to suit the sample size providing the ratio of HMDS to TMCS is kept between 4:1 and 1:1. The minimum volume of cyclohexane which can be used is 0.3 ml.
Chromatography

(a) The most commonly used liquid stationary phase for the glc of silylated compounds is SE-30, a non-polar liquid phase (see Table I, Literature Review) and this was tried first. However, it caused pyridine (in the pyridine/HMDS/TMCS silylation system, Results, I) to tail in the glc. The peaks came out on the solvent slope and this makes peak quantification difficult. In addition, SE-30 did not resolve glucose and galactose and therefore some other columns were tried.

The next two attempts were forms of gas solid chromatography and consisted of modifying the surface of the solid support normally used for column packings, Chromosorb W. The first modification tried was silylating the surface of the Chromosorb W to remove active sites which bind compounds and cause tailing. Unfortunately, tailing of both the silylation solvent (pyridine) and the silylated samples occurred.

The next solid stationary phase tried was reported to give good resolution of Krebs acids in the glc\textsuperscript{57}. It consists of a monolayer of Carbowax 20M bonded to the Chromosorb W by high temperatures. It was found to be useful at first because it could resolve glucose and galactose but the column deteriorated quickly under temperature programming conditions. The sample peaks started to tail because the solid support was being pulverised by the temperature changes involved in temperature programming. This crushing created new (unmodified) surfaces on the Chromosorb W which were unsuitable for the glc of silylated compounds.

However, Carbowax 20M itself, used as a stationary liquid phase rather than being modified into a solid phase, gave resolution of
silylated glucose and galactose (Fig. 2) while these compounds came out as one peak on SE-30. Carbowax 20M also minimized pyridine tailing and was thus chosen over the other columns for subsequent chromatograms (see Silylation section IIIe).

(b) When G-6-P was first silylated (in the DMSO/TMCS/Ag⁺ system (Silylation section IIIc)), the silylated G-6-P peak came out near the maximum operating temperature of the Carbowax 20M column. The high temperature needed to elute the silylated G-6-P caused excessive bleeding of the column (loss of the liquid phase) which shortens the column lifetime and alters its properties. Although the visible result of column bleed i.e., baseline drift, could have been compensated for in our gas chromatograph by using a second (compensating) Carbowax 20M column, the problem of column deterioration was still present. Therefore, a search was made for a different polar column with a higher maximum operating temperature (>250°).

No liquid phase similar in nature to the very polar Carbowax 20M had a higher maximum temperature. However, there were other liquid phases classified as having intermediate polarity, between the successful Carbowax 20M and the unsuccessful SE-30 (see Table 1, Literature Review) with higher maximum operating temperatures available. These intermediate polarity columns had also been used in the literature reports with silylated simple sugars and were therefore tried.

XE-60, recommended by Ellis⁵⁸, was found to produce asymmetric glucose peaks in the glc (Fig. 9) and thus was judged not likely to be of general usefulness. SE-52, the most polar of the SE series of liquid phases⁵⁴ gave good chromatograms for silylated glucose and G-6-P (using
Figure 9: Glucose on XE-60.

Injector Temp.: 200°  Detector: 300°
Column: 5% XE-60 on 80/100 Chromosorb W
       6' x 1/4" glass
Column Temp. 130° to 225° at 4°/min.
Attenuation: 10 x 256

Sample: 10 mg glucose silylated in DMSO/HMDS/TMCS (1.0:0.2:0.1)
        3 μl injection.

Peaks: 1 solvent
       2 α-glucose
       3 β-glucose
       4 unknown
the DMSO/TMCS/Ag\(^+\) silylation system) and it was used in most of the subsequent tests. Because column bleed from SE-52 was very small, a compensating column was not considered necessary at this time.

When G-6-P was shown to be silylated in the DMSO/HMDS/TMCS/cyclohexane silylation system, (Silylation section IIIe(iii) above), OV-1 and OV-25 were tried for their ability to resolve silylated sugar phosphates. These stationary phases, which are similar to SE-30 and SE-52, respectively, are highly recommended by Pierce\(^46\) for general work with silylated compounds.

On OV-1, cyclohexane had a very high retention temperature which interfered with sugar phosphate analyses (cf. pyridine tailing). Despite a similar maximum operating temperature (300\(^\circ\)), OV-1 bled more than all of the other high temperature columns and it was not used for further work.

OV-25 appeared to give better resolution of the sugar phosphates than did SE-52 but the peaks obtained were asymmetric (Fig. 10). This, combined with a relatively high bleed rate and a higher retention temperature for cyclohexane (than SE-52) left SE-52 as the preferred liquid stationary phase.

After the DMSO/HMDS/TMCS proved useful as a silylating mixture, the Carbowax 20M column was re-tested. Figure 11 shows a silylated G-6-P sample chromatogrammed on the Carbowax 20M column. The multiplicity of peaks may be noted (not present on SE-52) as well as the small size of the sample peak(s). Thus, Carbowax 20M was not a suitable column for the glc of sugar phosphates. These observations may account for the apparent initial failures to silylate G-6-P (Silylation section III(b) above).
Figure 10: Sugar Phosphates on OV-25

Injector Temp.: 150°  Detector Temp.: 300°
Column: 5% OV-25 on 80/100 Chromosorb W
        6' x 1/4" glass
Column Temp. 140° to 280° at 4°/min.
Attenuation: 10 x 128

Sample: 2 μl each of sample containing 5 mg of F-6-P or G-6-P

Peaks: 1 solvent
       2 F-6-P
       3 G-6-P
       4 unknown

Note the asymmetry in the sample peaks and the delay before the solvent (cyclohexane) front.
Figure 11: G-6-P on Carbowax 20M

Injector port: 200°  Detector: 300°
Column:  5% Carbowax 20M on 80/100 Chromosorb W
        6' x 1/4" glass
Column Temp.: 130 to 250° at 4°/min and then isothermal at 250°
Attenuation: 10 x 64

Sample: 10 mg of G-6-P in DMSO/HMDS/TMCS/cyclohexane (1.0:0.2:0.1:1.0).
Chromatographed on SE-52 and found to give only one peak.
10 µl injection.

Peaks: 1 solvent
       2  G-6-P

All other peak identities unknown.
Attempts to substitute a 1/8" copper column for the 1/4" glass ones used above resulted in failure. This was due to column deterioration which caused the loss of sugar phosphates and restrictions in carrier gas flow, (see also fig. 8). Glass columns are therefore preferable.

(c) Figures 12, 13 and 14 show calibration plots for simple sugars and sugar phosphates. It can be seen in these graphs that simple sugars such as sorbitol, N-acetylglucosamine and glucose give reproducible plots passing through the origin. [GNH₂ (not plotted) which arose in one sample from GNH₂-6-P decomposition (Silylation section III(g) above) was identical to glucose and N-acetylglucosamine in its behaviour.]

Figure 15 is a continuation of the glucose calibration curve of Figure 14 to smaller sample sizes (achieved by repeated dilutions with cyclohexane). It can be seen that the curve will pass through the origin. The smallest amount of glucose detected was $3 \times 10^{-4}$ μg (equivalent to $1.8 \times 10^{-12}$ moles).

Early work in the quantitative calibration studies for G-6-P revealed that the G-6-P peak decreased in size with decreasing sample size until the peak disappeared into the baseline (Fig. 16) although the sample injection volume was 1 μl (Fig. 12). This disappearance of the G-6-P peak at low sample volumes would seriously limit the sensitivity of the glc assay technique. The two other column, XE-60 and OV-25, tested earlier but not used because of asymmetric sample peaks were re-tested to see if G-6-P also disappeared on these columns when small samples were injected. The results were similar to those observed with SE-52. (OV-25 and SE-30 also showed the "priming" effects reported by Sherman (see Literature Review).
Figure 12: Peak Height vs Sample Volume for Sorbitol, F-6-P and G-6-P

Injector Temp.: 150°  Detector: 300°
Column: 5% SE-52 on 80/100 Chromosorb W  
6' x 1/4" glass
Column Temp.: 140° to 280° at 4°/min.
Attenuation: varied

△ : sorbitol
□ : F-6-P
○ : G-6-P

Sorbitol data was taken using the sample of sugars used for Fig. 6. No change in the peak pattern occurred throughout the study.

F-6-P and G-6-P were in the same vial and silylated together (5 mg each) using DMSO/HMDS/TMCS/cyclohexane (1.0: 0.2:0.1:1.0).
Figure 13: Calibration Curves Part I

Injector Temp: 150°  Detector: 300°
Column: 5% SE-52 on silylated Chromosorb W
         6' x 1/4" glass
Column Temp.: 140° to 280° at 4°/min.
Attenuation: varied

Data is from two independently silylated sugar mixtures. Each mixture contained:

<table>
<thead>
<tr>
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<th>I</th>
<th>II</th>
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<tbody>
<tr>
<td>glucose</td>
<td>7.6 mg</td>
<td>7.3 mg</td>
</tr>
<tr>
<td>N-acetyl glucosamine</td>
<td>11.5</td>
<td>13.5</td>
</tr>
<tr>
<td>G-6-P</td>
<td>5.7</td>
<td>7.9</td>
</tr>
<tr>
<td>F-6-P</td>
<td>7.7</td>
<td>6.6</td>
</tr>
<tr>
<td>GNH₂-6-P</td>
<td>10.4</td>
<td>7.5</td>
</tr>
</tbody>
</table>

They were silylated in DMSO/HMDS/TMCS/cyclohexane (1.0/0.8/0.4/1.3)

○: N-acetyl glucosamine
□: F-6-P
●: N-Ac-GNH₂ duplicate
■: F-6-P duplicate

For smaller concentrations, (< 1 µg) dilutions of the sample were made with cyclohexane.

Peak heights are given as if the sample had been chromatogrammed at an attenuation of 10 x 1.
Figure 14: Calibration Curves Part II

See Legend for Figure 13 for chromatography and silylation data

- △: glucose
- ○: G-6-P
- ▲: glucose duplicate
- ●: G-6-P duplicate
Figure 15: Glucose Calibration Plot

See the legend for Figure 13 for chromatography and silylation data.

▲: continuation of the glucose duplicate from Figure 14

Smallest sample injected contained $3.2 \times 10^{-4}$ µg of glucose and gave a peak 1 cm high at an attenuation of 1 x 8.
Figure 16: G-6-P on SE-52

Injector Temp.: 150°  Detector: 300°
Column: 5% SE-52
6' x 1/4" glass
Column Temp.: 110° to 250° at 4°/min.
Attenuation: 10 x 64

Sample: 5 mg G-6-P silylated in DMSO/HMDS/TMCS/cyclohexane (1.0:0.2:0.1:1.0)

Chromatogram A: 5 μl injection
Peaks: 1 solvent
2 G-6-P
3 unknown

B: 2 μl injection

C: 1 μl injection

This figure is an almost exact copy of the original chromatogram. Recorder zero was adjusted each injection to avoid overlap.
As SE-52 was not unique as a liquid stationary phase in causing the loss of the G-6-P peak at low sample size, it was thought that the Chromosorb W (the solid support for the liquid stationary phases) might be the cause of the problem. Some Chromosorb W was deactivated by silylation (see Methods) before SE-52 was coated onto it and the column was repacked. Deactivation of the solid support did not significantly reduce sugar phosphate losses (Figs. 13 and 14). Chromosorb W was retained however, because it is the purest silicate solid support available and of these silicate supports it is also best able to resist the physical breakdown caused by repeated temperature programming.

Calibration plots for F-6-P and G-6-P are shown in Figures 13 and 14 respectively. F-6-P gives a reproducible linear plot but one which intersects the baseline at the 2 μg level. G-6-P also disappeared at the 2 μg level but the plot produced is neither linear nor reproducible. GNH₂-6-P is not shown (Silylation section III(g) above) but it had already proved itself a more sensitive compound than G-6-P. (All calibration plots had been done with an injection port temperature of 150°). Loss of sugar phosphates at the 2 μg level in the glc makes direct detection of sugar phosphates in yeast unlikely. (See Appendix I).

While the disappearance of peaks at small sample sizes for the sugar phosphates is a problem, it could be solved by labelling the sugar phosphates with ¹⁴C label together with "cold" carrier to both minimize radioactive label losses and increase peak size in the glc. Recovery of the radioactively labelled compounds using a fraction collector could provide data suitable for monitoring metabolic events. Other possibilities would be to increase yeast sample size or injection sample size.
It was therefore thought worthwhile to investigate the limitations of the standard silylation procedure when applied to yeast extracts.

**Yeast Studies**

(a) In order to study yeast metabolic systems, a fast collection and extraction procedure is required to capture the transient metabolic compounds. Since the sample will be silylated as part of the glc analysis procedure, materials not properly part of the sample (culture medium, cell walls, proteins, etc.) must be removed because these use up silylation reagents and introduce extra peaks on the glc. Nor must the collection and extraction procedures add extraneous material to the sample. Lastly, the procedure must allow drying of the sample because water reacts rapidly with the silylating reagents and will also degrade the silylated products.

A very fast collection procedure available for yeast was filtration using a Millipore filtration apparatus. Filtration allowed the rapid removal of the yeast from the relatively large volume of culture medium in which the yeast were grown. Washing of the yeast sample on the filter to remove the last traces of culture medium was not done because it was thought that this would take too long and some metabolites might be lost. Yeast culture volumes of between 2.0 and 30.0 mls were filtered on 25 mm, 0.45 μ millipore filters and then immersed in 3.0 ml of extraction reagent, shaken and/or sonicated and then centrifuged to remove the filter and cell debris. A sample was then removed and dehydrated before silylation was attempted (see also Methods).

Several extraction reagents were tried. Trichloroacetic acid (TCA) extraction was tried first because TCA can be removed from water
solutions with ether. It is also a volatile acid and possibly could be
removed during the freeze-drying of the sample. Filtered yeast were
immersed in 3.0 ml of 0.5 M TCA, shaken, centrifuged and a 2 ml sample
removed. The sample was extracted twice with ether (1.5 ml each time)
and frozen. Unfortunately, this did not remove all the TCA. The
residual TCA evaporated less rapidly than the ice and became concentrated
enough to melt the sample and cause decomposition. The sample when
dried was in the form of discoloured pellets and still had an odour of
TCA. Because of these difficulties, the TCA extraction method was
abandoned.

Perchloric acid extraction was tried next. This procedure was
designed as a (successful) prelude to enzymatic analysis\(^{30,60}\) rather
than to freeze drying. The filtered yeast was shaken with 3 ml of
0.5 M perchloric acid and centrifuged. A 2.5 ml sample of the super­
natant was treated with potassium carbonate and cooled to precipitate
the perchlorate ion as potassium perchlorate; this also neutralized
the solution.

Two ml perchloric acid extract samples freeze-dried properly but
left approximately 30 mg of solids (mainly salts) in the sample.
Although it was not known how the presence of these salts would affect
the silylation reaction, enough silylation reagent (DMSO/HMDS/TMCS/
cyclohexane; 1.0:0.6:0.3:1.0) was added to silylate 30 mg of solid and
a sample of the resulting upper phase was then chromatogrammed (Fig.
17). This procedure was not completely successful as will be described
below.
Figure 17: Chromatography of a Perchloric Acid Extracted Yeast Sample

Injector Temp.: 150°  Detector Temp.: 300°
Column: 5% SE-52 on silanized 80/100 Chromosorb W
           6' x 1/4" glass
Column Temp.: 110° to 290° at 4°/min.
Attenuation: 10 x 8
Injection Sample Size: 10 µl
Culture Sample Size: 10 ml

Extraction Conditions: see text
Silylation Conditions: DMSO/HMDS/TMCS/cyclohexane (1.0:0.6:0.3:1.0)
Sample sonicated until dissolved.

Chromatogram A: Reagent blank containing only the silylating reagents

Chromatogram B: Yeast Extract
Peaks: 1 solvent
       2 α-glucose (from the culture medium)
       3 β-glucose (from the culture medium)
       4 peak attributed to yeast
       5 peak attributed to yeast

Identity of all other peaks is not known.
Ethanol extraction is a routine method for the extraction of sugars from plant material and this technique was investigated as a possible alternative to the acid extractions. An initial test showed that G-6-P dissolves readily in slightly acidified 80% ethanol and the following method was adopted. The filtered yeast were immersed in 3.5 ml of 80% ethanol acidified with 5 μl of 5N HCl and the mixture sonicated in a water bath (see Methods). After centrifugation a 3.0 ml sample was taken and evaporated overnight in vacuo to a constant weight. A plot of yeast extract weight versus culture sample volume (Fig. 18) yields a straight line which almost intersects the origin. Ethanol extraction was therefore used for subsequent yeast studies in preference to the perchloric acid method.

The effect of the ethanol extraction procedure on the sugar and sugar phosphate peak pattern was investigated. Solid glucose, F-6-P and G-6-P were dissolved in 0.5 ml of water and allowed to stand for four hours. Then 2 ml of absolute ethanol was added and the mixture dried in vacuo overnight to simulate the extraction procedure. When these samples were silylated (with DMSO/HMDS/TMCS/cyclohexane system) no visible change occurred in the sample peak pattern compared to freeze-dried samples allowed to mutarotate in water only. Thus ethanol extraction does not appear to affect the sample peak patterns in any significant way.

(b) Figure 19 shows a silylated, ethanolic yeast extract sample chromatogrammed in the glc. (The sample silylated readily in the DMSO/HMDS/TMCS/cyclohexane system.) The peaks in the figure are identified in the legend. The positions of F-6-P and G-6-P peaks as determined by co-chromatography have been superimposed to show where
Yeast samples were harvested onto a Millipore filters which were immersed in 3.5 ml of 80% ethanol, sonicated for 5 min. and then centrifuged. 3.0 ml of the supernatant was transferred to a pre-weighed vial and dried in vacuo to constant weight.

The vertical bars represent the inaccuracy inherent in weighing samples on a balance (±0.1 mg) and the horizontal bars represent the potential error in pipetting the samples.
Figure 19: Chromatogram of Alcohol Extracted Yeast Sample.

Injector Temp.: 150°  Detector Temp: 300°
Column: 5% SE-52 on Silanized 80/100 Chromosorb W 6' x 1/4" glass
Column Temp.: 140° to 290° at 4°/min.
Attenuation 10 x 4
Injection Sample size: 10 µl

Extraction Method: see text

Peaks: 1 solvent
2 α-glucose (from culture medium)
3 β-glucose (from culture medium)
4 F-6-P position determined by co-chromatography
5 peak attributed to yeast
6 peak attributed to yeast
7 G-6-P position determined by co-chromatography
8 unknown
9 peak attributed to yeast
these compounds would appear if present in detectable amounts (see also Appendix I). It can be seen that the extraction procedure generates glc peaks which would interfere with the direct detection of sugar phosphates in the sample. This chromatogram can be compared to the perchloric acid extracted sample (Fig. 17). The yeast peaks are similar but the contaminants are of a different size and type. The previous chromatograms (Figs. 2 to 10) were derived using solid samples of pure compounds. The concentration of the silylated product was high and easily detected in the glc (attenuation usually 10 x 128). In order to detect metabolic intermediates directly in the yeast extracts, the glc sensitivity must be increased (attenuation 10 x 4) as well as sample size (from 3 - 5 μl to 10 μl) to the limits of usefulness. When the attenuation was lowered, i.e., sensitivity increased, many spurious peaks which had previously been hidden in the base line were revealed and found to rival the sample peaks in size.

Figure 20 shows a plot of peak height versus culture sample size for two peaks which may be attributed to the yeast and one peak considered to be part of the background. It can be seen that the height of the peaks attributed to yeast increase with increased sample size while the background peaks are more or less constant in size. All of the other peaks in Fig. 20 can similarly be attributed to the background except for peak 9 which lies outside the area of interest.

(c) Figure 21 shows the sources of some of these spurious peaks. Many are present in the reagent blank (containing only DMSO/HMDS/TMCS/cyclohexane; 1.0:0.05:0.03:0.3) and some are contributed by the culture medium.
Figure 20: Plot of Yeast Peak Height vs Culture Sample Size.

Chromatography Conditions: see legend Figure 19

Data taken from a duplicate experiment similar to the experiment which yielded the chromatogram in Fig. 19.

- : Yeast peak corresponding to peak #5 in Fig. 19
- : Yeast peak corresponding to peak #6 in Fig. 19
△: peak corresponding to unknown peak #8 in Fig. 19
Figure 21: Sources of Background Contamination.

Chromatography Conditions: see legend Fig. 19.

The culture medium blanks were obtained by passing 5 ml of medium through a Millipore filter and then treating the filter as if it carried a yeast sample. Culture medium was minimal medium glucose.

Chromatogram A: Reagent blank containing only the silylation reagents.

B: Medium blank before yeast have grown in it.

C: Medium blank after yeast had been grown in it.

Peaks:
1 solvent
2 α-glucose
3 β-glucose

All other peak identities unknown.
The sources of background peaks in the reagent blank were investigated further (Fig. 22). Chromatograms for DMSO, HMDS, TMCS and the extraction blank are shown. Chromatograms for ethanol and cyclohexane are not shown because these solvents had been purified and gave no extraneous peaks in the region of interest (see Methods). It can be seen that there are peaks in the blank which are not present in the original reagents. (For those peaks present in both the blank and a reagent, note the difference in sample sizes.) Some of these peaks were found to increase dramatically in size if the sample was shaken, or exposed to air or left longer than 24 hours at room temperature or refrigerated. Thus sonication in a septum stoppered vial was adopted as the mixing procedure in the silylation step and the samples were chromatogrammed as soon as possible after silylation.

Contamination from other sources had previously been minimized by using all-glass syringes for handling the reagents, by the use of teflon-faced silicone rubber septa on the vial and purifying reagents whenever possible (see Methods). Other batch lots of each of the silylation reagents (HMDS and TMCS) were tested and similar results were obtained (Appendix II). Despite all of these precautions, the level of contamination shown in Figure 21 for the blank is the lowest ever achieved in this work.

(d) In the chromatogram of the yeast extract (Fig. 19) the yeast sample peaks occur on the solvent slope before column bleed becomes a factor. A compensating column in the glc would not improve sensitivity in this case. A reduction of the solvent slope however might allow higher sensitivity.
Figure 22: Sources of Background Contamination (cont'd.)

Chromatography conditions: see legend Fig. 19

Chromatogram A: 10 µl DMSO
B: millipore blank containing an unused millipore filter treated as if it had contained a yeast sample. Reagent volumes add to the sample. HMDS: 0.05 ml TMCS: 0.03 ml cyclohexane: 0.3 ml DMSO: 1.0 ml
a 10 µl injection of the sample layer was made.

C: 7 µl HMDS

D: 4.1 µl TMCS
Ellis\textsuperscript{50} reduced the interference from DMSO and excess silylating reagents by washing the upper layer containing the sample of silylated simple sugars with water. In our work, however, the silylated sugar phosphates were instantly hydrolysed giving only a broad low temperature peak in the glc.

Another attempt was made to reduce the solvent slope by freezing the DMSO (m.p. 19°C) out of solution but this had no effect on the solvent slope.

Since direct detection of sugar phosphates in yeast is unlikely because of the disappearance of the sugar phosphates in the glc at small sample size (Chromatography section IIId above) and direct interference from peaks contributed by the yeast, the next step in perfecting this technology might have been the application of radioactive tracers to this work. The addition of cold carrier to radioactive extracts would circumvent the detection problems encountered above (section III(b)), by providing large uniform peaks easily detected at high attenuation (see Fig. 11) which could subsequently be collected as fractions from the glc. However, perfecting this technique would be a major project and it was decided that it was outside the scope of this thesis.

\textbf{Summary}

This work has resulted in:

(i) the development of a new reproducible method for the simultaneous silylation of sugars, sugar phosphates and other sugar derivatives at room temperature in a one step procedure.
(ii) a chromatography column (SE-52) has been investigated and found useful and reliable.

(iii) a new procedure for the extraction of soluble material from yeast was independently developed.

In order for the technique to become practical as a method for direct assay of metabolites in yeast, the following difficulties must be overcome:

(a) loss of sugar phosphates at low concentration (less than 2 µg per injected sample).

(b) contamination from the original reagents.

(c) masking due to the large solvent slope at low attenuation.

(d) interference from the relatively large yeast peaks detected above.

Many of the above difficulties could be overcome by using an indirect method of increasing sensitivity, i.e., using radioactive tracers together with cold carrier added to the sample before extraction.
CHAPTER 5
DISCUSSION

The preliminary work in this thesis (Silylation I and II) with the pyridine or DMF/HMDS/TMCS silylation system was an attempt to reproduce or improve upon the work reported in the literature. The original paper on silylation of sugars by Sweeley et al.\textsuperscript{45} reported no difficulty in silylating a wide variety of sugars including glucosamine. My failure to silylate glucosamine is, however, not unusual. It is believed that the hydrochloric acid in the glucosamine hydrochloride (as supplied) inhibits silylation\textsuperscript{1,61}. Silylation of glucosamine can be carried out by heating in the silylation mixture, removing the HCl beforehand by ion exchange resin\textsuperscript{62}, or by using a tenfold excess of silylating reagents\textsuperscript{63}. The silylation reaction in pyridine is also sensitive to water in the sample and carefully dried sugar phosphate samples\textsuperscript{3a} may have helped Hasbuzume and Sasaki silylate their sugar phosphate samples by heating\textsuperscript{3b}. In my work, sugar phosphates were used as supplied without further treatment.

The pyridine BTSA/TMCS silylation system of Sherman et al.\textsuperscript{6} resulted in multiple peaks for glucose and glucosamine as Pierce indicated would happen\textsuperscript{46}. No silylation of sugar phosphates occurred using the usual order of adding the reagents, pyridine first then the silylating reagents (Silylation III(a)). Sherman lists the reagents in opposite order to the above and may have added the BTSA and TMCS first but he does not explicitly specify this order. If this ordering
of the reagents were the case, the silylation reaction would be dangerously exothermic. Sherman also reported that biologically derived samples required longer than three hours to silylate with no explanation offered for this result. These results, together with those obtained in this study show that pyr/HMDS/TMCS system is not a workable silylation system for sugars and sugar phosphates.

The apparent silylation of G-6-P with DMF/BTSA/TMCS (high temperature peaks, Fig. 3) is more probably due to the spurious peaks Sherman found in his samples than to silylation of G-6-P. Glucose-6-P did not give this pattern in any other sample and the pattern resembles more closely the contamination peak patterns which have occurred elsewhere (e.g., Appendix II, A: note the change in attenuation from fig. 3). Also, this sample was chromatographed on Carbowax 20M which is not suitable for the glc of sugar phosphates (Chromatography (b)).

It is believed that trans-silylation occurs between the silylated sugar phosphate and the silylated hydroxyl group of the Carbowax 20M so that this phosphate ester bond traps the sugar phosphate in the liquid phase. Comparison of G-6-P peak size and pattern can be made between Figs. 4 and 11. In fig 4, the Carbowax 20M column had been used repeatedly during the early attempts to silylate and chromatogram G-6-P and this column was probably conditioned by having had all of the ester forming sites saturated with G-6-P esters. In Fig. 11, the column was not thoroughly conditioned by repeated injections of silylated G-6-P samples. This is the most likely reason why Carbowax 20M failed as a liquid phase in this work (Chromatography (b)).
The conditioning of the Carbowax 20M column explains why I obtained a G-6-P peak with the DMSO/TMCS/Ag⁺ silylation system and not with the original DMSO/HMDS/TMCS system of Ellis⁵⁰. The DMSO/TMCS/Ag⁺ silylation system resulted in reproducible, though unstable, silylation of G-6-P and allowed an investigation of other stationary liquid phases. The final choice of SE-52 (Chromatography (a) and (b)) was based on its thermal stability, the fact that it resulted in symmetrical peaks for sugars and sugar phosphates and its ability to resolve complex sample mixtures (figs. 7 and 8). The choice was also somewhat fortuitous as the SE-52 columns did not need "priming" nor did I encounter the other difficulties reported by Sherman (see Literature Review). However, my work with OV-25, SE-30 and SE-52 in a copper column did encounter the same difficulties reported by Sherman (Chromatography (b) and (c)).

The choice of SE-52 led back to the DMSO/HMDS/TMCS system and eventually to the DMSO/HMDS/TMCS/cyclohexane silylation system. This used partitioning of the silylated sample into an upper cyclohexane liquid phase as the driving force (Silylation section IIIe(ii)) rather than precipitation of Cl⁻ ions (Silylation III(c)) or heating as required with the Ellis system (Silylation IIIe(i)). This was especially apparent with DMF as solvent where the silylation time for G-6-P is reduced from 18 hours to one hour when cyclohexane is added to the silylation mixture. This mild silylation technique allows the silylation of G-1-P and my work indicates that the failure of other workers to silylate G-1-P can be attributed to their harsh silylation conditions³,⁵ which would cause decomposition of the G-1-P sample (cf. Silylation section). Adding cyclohexane also adds needed volume to the upper liquid phase when silylating small samples. Thus my
DMSO/HMDS/TMCS/cyclohexane system is better than other silylation systems reported in the literature (see also Silylation IIIe(ii)).

This discussion of the Silylation section of the Results ends with a comment on the failure of GNH₂-6-P to silylate reproducibly in mixed sugar and sugar phosphate samples (Silylation III(g)) as it silylated easily and reproducibly although relatively slowly as a pure sample. GNH₂-6-P would be the last of the compounds in the mixture to silylate (see Table II) and the HCl (from TMCS) could complex with the amine to inhibit silylation as happens with GNH₂ (above). It is likely that only an excess of silylating reagents is required to effect complete silylation of GNH₂-6-P.

The selection of SE-52 as the stationary liquid phase in the Chromatography section is discussed above. Calibration plots on this type of column (figs. 12 to 15) revealed that less than 1.8 x 10⁻⁶ μmoles of glucose can be detected (fig. 15) by the flame ionization detector on the glc although the actual limit to the sensitivity would be restricted to ≈0.05 μmoles by the presence of background peaks in the silylation mixture (figs. 21 and 22). These peaks become apparent when a change is made from high concentration standard samples to low concentration biological samples. These background peaks were always there but weren't large enough to interfere with earlier chromatograms (figs. 6 and 7) because of the large differences in sensitivity required with the glc between standard and biological samples.

Loss of sugar phosphates at the 2 μg level (figs. 13 and 14) severely limits sensitivity for this class of compounds. Sherman's priming effect and his explanation for column failures (see Literature Review) would indicate that he too must have expected losses of sugar...
phosphates though he reported no studies on this. Eisenberg and Bolden reported losses of 50% for G-6-P but did not speculate on the cause of this large loss of sample.

In my calibration studies, a new SE-52 column was used which neither needed "priming" nor had had an opportunity to build up decomposition products which might catalyse further decomposition. It appears likely that some decomposition of the sugar phosphates occurs as a result of the high temperatures required to vaporize them in the injection port (e.g., GNH₂-6-P in silylation section e(i)) or to elute them during chromatography. It may not be just coincidence that the earlier the sugar phosphate exits from the gc, the more stable it appears to be; i.e., F-6-P > G-6-P > GNH₂-6-P and G-1-P > FDP (Chromatography section (d)) the latter pair being aldose-1-phosphates and not directly comparable to the first three. This could be tested by preparing dimethylsilyl derivatives (the necessary reagents having recently become available) which are more volatile than the trimethylsilyl derivatives used in this work. More inert solid supports, e.g., teflon or silver coated Chromosorb W could also be tested to see if the levels of decomposition are similar.

The Yeast Studies were undertaken to develop a fast extraction procedure which would give samples suitable for silylation in preparation for gc work. I found that acidic ethanol extraction was as good as perchloric acid extraction (Yeast studies section (b)) and it left no residue after drying as did the TCA or perchloric acid extraction procedures. Recently, other workers have begun to use ethanol extraction for yeast and E. coli studies. The peaks contributed to the sample by the yeast (fig. 19) are not a major problem because the losses of the sugar phosphates at the two mg level would necessitate
the use of radioactive tracers and enough "cold" carrier to give a large peak if metabolic studies were to be carried out. However, the tendency for the sugar phosphates to decompose could cause cross contamination of other peaks and this might restrict the usefulness of this technique.

The search for yeast sample peaks in the glc also revealed that contamination occurs from the silylation reagents and the silylation reaction itself (Figs. 21 and 22) and Appendix II which is detected when high sensitivity is employed. The background peaks will restrict studies into the natural occurrences of sugars as carried out by Holligan. These background peaks, which are discussed extensively by Sherman could also be the cause of Eisenberg and Bolden's "anomalous peak pattern". As an investigation of these background peaks would be a major study in itself, I could only report under what conditions they appear (Yeast Studies (c)) and ways to merely minimize them but not eliminate them (see Methods).

In conclusion, this thesis reports the development of a new silylation procedure which allows fast, easy silylation of a range of substances (Table II) which is mild enough to silylate relatively unstable compounds. It has answered some questions concerning the simultaneous silylation and chromatography of sugar phosphates (cf. Eisenberg and Bolden) and has indicated the general levels of sensitivity obtainable with this silylation technique for sugars (cf. Holligan) as well as sugar phosphates. My silylation technique would probably be of immediate application in polysaccharide work or glycoprotein studies (for both sugar and amino acid composition) or, in conjunction with radioactive tracers it might be useful for metabolism studies.
BIBLIOGRAPHY


     (b) Ibid., pp. 199-203.


(10) Ibid., p. 876.


(12) Malaprade, L., Ibid.

(13) Ref. 9, p. 872

(15) Ref. 9, p. 889.


(17) Ref. 9, p. 927.


(21) Benson, A.A., see ref. 18, p. 110-129.


(24) Ashwell, G., see ref. 18, p. 73.


(26) Foster, A.B. in ref. 20, p. 21.

(27) Ref. 9, p. 943.


(31) Ibid., p. 551.
(33) Ref. 30, p. 134.
(59) Catalogue information supplied by Varian Aerograph Corp., Walnut Creek, Calif.

This section is a calculation of the amounts of F-6-P or G-6-P expected in a typical yeast sample.

From Ball et al.\textsuperscript{55}, the amount of F-6-P or G-6-P in \textit{S. cerevisiae} is approximately 2.1 picomoles/mg protein.

From A. Ball and L. Maheshwari (unpublished results), a yeast culture with an OD of 0.83 at 415 nm contains 1.88 mg protein/ml.

A reasonable sample size which allows rapid sampling by Millipore filtration is 10 ml of culture medium.

Molecular weight of G-6-P or G-6-P is 282.1 picograms/picomole.

Multiplying all of these factors together, the expected sample size for F-6-P or G-6-P =

\[(2.1 \text{ pmole/mg protein}) \times (1.88 \text{ mg protein/ml}) \times (10 \text{ ml}) \times (282.1 \text{ pg/pmole})\]

\[= 11,100 \text{ picograms or 0.011 \mu g.}\]

F-6-P and G-6-P decompose in the glc and are lost at the 2 \mu g level (Silylation section (c)). Thus there is not enough sugar phosphate in a 10 ml yeast culture sample to be detected by the glc even if there were no interference by background peaks in the sample (Figs. 19, 21 and 22).
Appendix II
Figure 23: Chromatography of Different Batch Lots of Silylating Reagents.

Chromatography Conditions: see legend Fig. 19

Chromatogram A: 4.2 μl of TMCS used throughout this work.

B: 4.2 μl of another batch lot of TMCS.

C: 7 μl of HMDS used throughout this work.

D: 7 μl of another batch lot of HMDS.