# A STUDY OF THE ALDOPENTOSES AND FUCOSE IN HUMAN URINE BY THIN-LAYER CHROMATOGRAPHY

by

M. Q.-K. Talukder M.B.,B.S. (Dacca), DIP. NUTR. (London).


Thesis presented for the Degree of Doctor of Philosophy of the University of Edinburgh in the Faculty of Medicine

1972

TO THE MEMORY

of a great teacher and friend

Dr. D. J. Bell

who, throughout the years of this work

has been a source of unfailing inspiration

C O N T E N T S

ACKNOWLEDGEMENTS

I am permanently indebted to Dr. D.J. Bell who died suddenly on 3rd April 1972. A man of great warmth and extraordinary sympathy he appreciated my initial inexperience and under his guidance I was able to learn all I know of scientific techniques. This thesis owes much to him. My sense of personal loss at his death is very great. I also have a deep sense of gratitude to Dr. R. Passmore for his help in starting this work and for continued interest and guidance.

I am indebted to Dr. S.H. Shariff and Dr. N. Powers for their help and valuable criticism during this work and preparation of the manuscript. Without my wife's cooperation and understanding this work should never have been possible and I am grateful to her.

I wish to thank Dr. J.S. Robson for arranging urine collections from renal failure patients; all those who provided samples for analyses; Dr. G.H. Begbie who gave statistical advice; all members of the staff of the Physiology Department for their help and cooperation; and Miss J. Gray and Mrs. A. Falconer for typing this thesis.

I am deeply indebted to the Government of the Peoples Republic of Bangladesh for financial support.

This work was carried out in the Edinburgh University Department of Physiology. I am grateful to Professor W.E. Watson for providing me with the facilities in a stimulating environment.

SUMMARY

SUMMARY

1. The thesis describes an investigation into the presence and quantities of four free, monosaccharides, L-arabinose, D-xylose, D-ribose and (presumably L-) fucose in the urine of healthy human subjects and of patients suffering from renal failure.

2. Two new quantitative analytical procedures based on one-dimensional thin-layer chromatography have been devised whereby it has become possible for the first time rapidly (3 h) to separate and measure at microgram levels, arabinose, ribose, xylose and fucose from the numerous other sugars present in urine from human subjects. On thin-layers of Kieselguhr G buffered with sodium acetate (acetate plate), arabinose and ribose are measured. On thin-layers of Kieselguhr G buffered with sodium dihydrogen phosphate (phosphate plate), fucose is determined. Xylose is measured by difference between ribose on an acetate plate and xylose plus ribose on a phosphate plate. Previously, only one complicated technique, occupying 5 days for the paper chromatography was available (Date, 1958a, 1958b, 1958c and 1966).

3. The spraying reagents used to colour the sugar spots can detect qualitatively to the lowest limit of 0.5 μg of each sugar on the devised t.l.c. systems. The new PABA reagent visually distinguishes different sugar classes on the both t.l.c. systems. This reagent may be employed for quantitative measurements whereby linear relationship holds upto 30 μg for recovered colour of xylose

from acetate plates and upto 80 ug for fucose from phosphate plates. Using the p- anisidine hydrochloride staining reagent, quantitative spectrophotometric determination of the extracted sugar spots is possible to the lowest limit of 2.5 µg for each sugar; linear relationship holds upto 80 µg for each sugar on either plate.

4. Preparatory to chromatography, deionisation of urine samples is obligatory and accomplished by means of ion-exchange resins Amberlite IR-120 ($H^+$) and Amberlite IRA-400 (acetate) using two glass columns in tandem.

5. Specificity is conferred on the estimates by the comparable behaviours of the urinary aldopentoses and fucose so estimated and the authentic sugars on the thin-layer chromatograms.

6. The separation of the sugars on the devised thin-layers is always reproducible. Quantitative reproducibilities (coefficient of variations) in a septuplicate analysis of the same urine sample show arabinose as 13.1%, xylose 15%, ribose 7.2% and fucose 10.2%. The mean±S.D. recoveries of 10-30 µg of arabinose, xylose, ribose and fucose added to the same urine samples in six experiments are 93%±15% (range 80-113%), 101%±41% (range 50-166%), 95%±9% (range 85-110%) and 95%±14% (range 80-110%) respectively.

7. All urine samples of six fasting healthy males and six fasting healthy females invariably contain arabinose, xylose, ribose and fucose. These sugars must presumably originate in endogenous processes. There is no significant difference in the excretion between the corresponding sugars in fasting state in two sexes. The

mean fasting rates of excretion (μg/min) of the sugars for the twelve subjects are 13.3±3.4 (arabinose), 8.5±1.9 (xylose), 5±1.5 (ribose) and 16.6±5.6 (fucose).

8. Consuming an ample diet, free as far as possible from known sources of aldopentoses, healthy subjects at three different periods of the day excrete the sugars at rates unchanged from those in the fasting state. It is suggested that these sugars are excreted at constant rate independently of the urine volume.

9. On diets potentially rich in aldopentoses, the subjects of (8) show increased rates of excretion of aldopentoses, mainly that of xylose and less so of arabinose. Noteworthy is the fact that rates of excretion of both ribose and fucose were not increased.

10. Urines from all six patients with various forms of renal failure repeatedly show the same picture. Ribose is always excreted at the lower end of the normal range while arabinose, xylose and fucose, if detectable, are excreted only in unmeasurable amounts.

11. The results are compared and discussed with other workers' findings. In so far as any reasonable suggestions can be made, the excretion of these four monosaccharides in health and in renal failure are discussed in the light of our knowledge of the somatic origins and metabolism of arabinose, ribose, xylose and fucose. The prospects of employing this technique and the measurements of these sugars in certain diseases are indicated.

# CHAPTER 1

# INTRODUCTION

# INTRODUCTION

This thesis describes new methods using thin-layer chromatography for determining the excretion rates of microgram quantities of four free, monosaccharides, L-arabinose, D-ribose, D-xylose and (presumably L-) fucose in the urine of healthy human subjects, fasting or otherwise. The excretion rates of these sugars in the urine of patients suffering from renal failure are also considered. The following brief account explains how this study came to be undertaken.

Pentoses have been suspected as a constituent of urine for a long time. Salkowski & Jastrowitz (1892), using crude qualitative tests, first detected what they considered to be pentosuria in a morphine addict. The "pentosuria" persisted after the patient stopped taking the drug and the subject might have been a L-xylulosuric (cf. Greenwald, 1930; Lasker et al., 1936). This condition is a benign inborn error of metabolism, believed to be confined to the Jewish race (Garrod, 1909).

Johnstone (1906) who subsequently became Professor of Obstetrics and Gynaecology in Edinburgh University, working in Prague and stimulated by Professor Von Jacksch and by the work of Salkowski & Jastrowitz (1892) discovered that consumption of fruits and their juices caused an 'alimentary pentosuria'. He used the phloroglucinol-HCl qualitative test for pentoses (Wheeler & Tollens, 1889) in all his experiments, including those on four subjects

examined after injecting therapeutic doses of morphine; "pentosuria" was observed in all four cases. This positive result, attributed by Johnstone to urinary pentose must in fact have been due to the presence of morphine glucuronosides (Wood, 1954; Oguri et al.,1970) since the strongly acidic Tollens' reagent would hydrolyse glucuronosides and reacts with hexuronic acids (Dische, 1962a). Johnstone's paper (1906) became a classic but at the same time led to subsequent misconceptions on the dietary origin of urinary aldopentoses.

While many later workers, e.g. Harding & Selby (1931) and Harding et al. (1936), using yeast fermentation and the formation of osazones, showed that normal fasting urine contained small quantities of non-fermentable as well as fermentable sugars, King's (1942) attempt to isolate from human urine a sugar responsible for the growth of a urinary strain of Esch. coli gave inconclusive results. Since D-ribulose and D-arabinose yield the same osazone as D-ribose, the controversial detection of ribosuria in muscular dystrophy (Orr & Mirot, 1952; Drew & Selving, 1953; Walton & Latner, 1954) by osazone formation could not be confirmed or refuted in the absence of more specific analytical methods.

Measured by the method of Roe & Rice (1948), total aldopentose in rat's urine was found to increase at low environmental temperatures and after thyroid administration (Coover et al.,1950). By the same method Tower et al. (1956) measured the total aldopentose in 230 samples of 24 h urines from 55 patients (on fruit free diets) with and

without muscular dystrophies. A significant increase in the excretion of total aldopentose in myopathies was observed.

In 1967, D.J. Bell and R.E. Cull and later in 1968, the present author examined urines from fasting subjects using Dische & Borenfreund's (1957) quantitative modification of Tollens' reagent; they found aldopentoses in all of numerous samples (Bell et al., 1972; this paper is appended to the thesis). Although the method gives weak reactions with glucuronic acid and glucuronolactone, it became apparent that there was aldopentose fraction whose nature should be investigated.

Eastham (1949) was the first to use paper chromatography to obtain evidence of the presence in human urine (of undescribed origin) of glucose, and xylose and mentioned the possible presence of rhamnose, a number of unidentified "fast runners" and a uronic acid.

Besides several unidentified sugar 'spots', Montreuil & Boulanger (1953), by paper chromatography of non-fasting human urines found 'spots' corresponding to arabinose, xylose and fucose, as well as glucose, fructose, mannose and sucrose. Rhamnose and galactose were observed in very feeble traces while ribose appeared rarely, if at all on their chromatograms. These authors were the first to deionise their samples by means of resins, a procedure which subsequent workers have found to be absolutely essential.

White & Hess (1956), after deionising urines by a mixed bed of resins, prepared concentrates suitable to detect small amounts of

sugars by a two-dimensional paper method. Their results on 15 non-fasting healthy subjects, 11 cases of muscular dystrophy and a single healthy subject on a fruit-free diet, usually showed the presence of arabinose, ribose, xylose and fucose, and a number of other monosaccharides. By "inspection" the single subject on a fruit-free diet, appeared to have lower urinary concentrations of all the sugars detected. Ribose and fucose were not always detected in every urine sample.

At the same time, Tower et al. (1956) examined by two dimensional paper chromatography in deionised urine from non-fasting patients on fruit-free diets, with or without muscle disease, and found glucose, xylose, arabinose and ribose in the majority of cases. A few exhibited spots corresponding to lactose, galactose, deoxyribose and fructose. "Semiquantitation" of the aldopentose showed significantly higher excretion of ribose in muscle disease in contrast to the findings of Ronzoni et al. (1955) and White & Hess (1956).

In the observations recorded above it is clear that in no instance could the urine samples be considered to be collected in the fasting state and therefore the findings gave no indication that any of these sugars found might be endogenous in origin.

Date (1958b) then examined two samples of his own urine in the fasting states by careful but time-consuming two dimensional paper chromatography (which occupied five days) and quantitative

spectrophotometric measurements, (Date, 1958a). He also identified both L-arabinose and D-xylose. In his original experiments he failed to detect ribose and fucose although later he described procedures (Date, 1958c) which could reveal both sugars. Eight years later under the title "The excretion of lactose and some monosaccharides in the urine in certain pathological conditions", Date (1966) measured the urinary aldopentoses and fucose in healthy fasting subjects and in cases of diabetes mellitus, cirrhosis of liver, infectious diseases, hyperthyroidism, and coronary occlusion. Because of the emphasis of the title of Date's (1966) report on 'Lactose' this paper escaped my attention until the present work, in a closely related field, was well under way.

From the foregoing information it was considered that a thin-layer chromatographic technique which would be quick and probably of greater resolution should be attempted in order to separate and if possible measure arabinose, xylose and ribose in human urine under various conditions, especially in fasting and in certain diseases and results might reveal the possible endogenous sources of these sugars.

While the technique for the quantitative analysis of arabinose, xylose and ribose was being developed (Bell & Talukder, 1970) and applied to urine it was found by a dichromatic spraying reagent (Bell & Talukder, 1970) that another sugar invariably

coincided with the xylose spot in all urine samples examined. This was found to be fucose, a deoxyhexose known to occur widely in mammalian tissue components etc. (Table 2). This sugar was also measured.

# CHAPTER 2

# ALDOPENTOSES AND FUCOSE IN MAMMALIAN METABOLISM

A. Occurrence of aldopentoses and fucose in mammalian tissues.

B. Metabolism and excretion of aldopentoses in man.

C. Fucose in mammalian metabolism.

D. Availability of aldopentoses and fucose to the human body from exogenous sources.

# A. OCCURRENCE OF ALDOPENTOSES AND FUCOSE IN MAMMALIAN TISSUES

## I. Arabinose, xylose and fucose

The occurrence of free arabinose and xylose has not been reported in mammalian tissue except in urine (this thesis). Fucose has been reported to be present in free form in human plasma only by Horning & Horning 1970. The L-, D-, and L- isomers respectively of these sugars were found as structural components of certain peptidoglycosaminoglycuronoglycans (GGG) and peptidoglycans. The structure, function and occurrence of these substances have been recently reviewed by Sharon (1966), Kent (1967), Ginsberg & Neufeld (1969) and Spiro (1970).

At present the role of xylose has been clearly defined only with regard to the acidic GGG in which the pentose serves as the point of linkage between carbohydrate and protein. The following table shows the pattern of linkages involving xylose.

TABLE 1

Fragments isolated from chondroitin 4-sulphate glycopeptides by partial acid hydrolysis (modified from Roden 1969).

1. O-β-D-Xylosyl-L-serine
2. 4-O-β-D-galactosyl-D-xylose
3. 4-O-β-D-galactosyl-O-β-D-xylosyl-L-serine
4. O-β-D-galactosyl-(1⟶3)-O-β-D-galactosyl-(1⟶4)-D-xylose

Roden (1969) suggested that the presence of a single xylose residue in a key position in the chrondroitin-4-sulphate-protein complex might have some specific structural and metabolic function, important for the complex as a whole. While it is beyond the scope of this thesis to go into details on the biosynthesis, occurrence and degradation of the sugars in mammalian tissue mucosubstances, schematic pathways of biosynthesis involving D-xylose are reproduced in fig. 1.

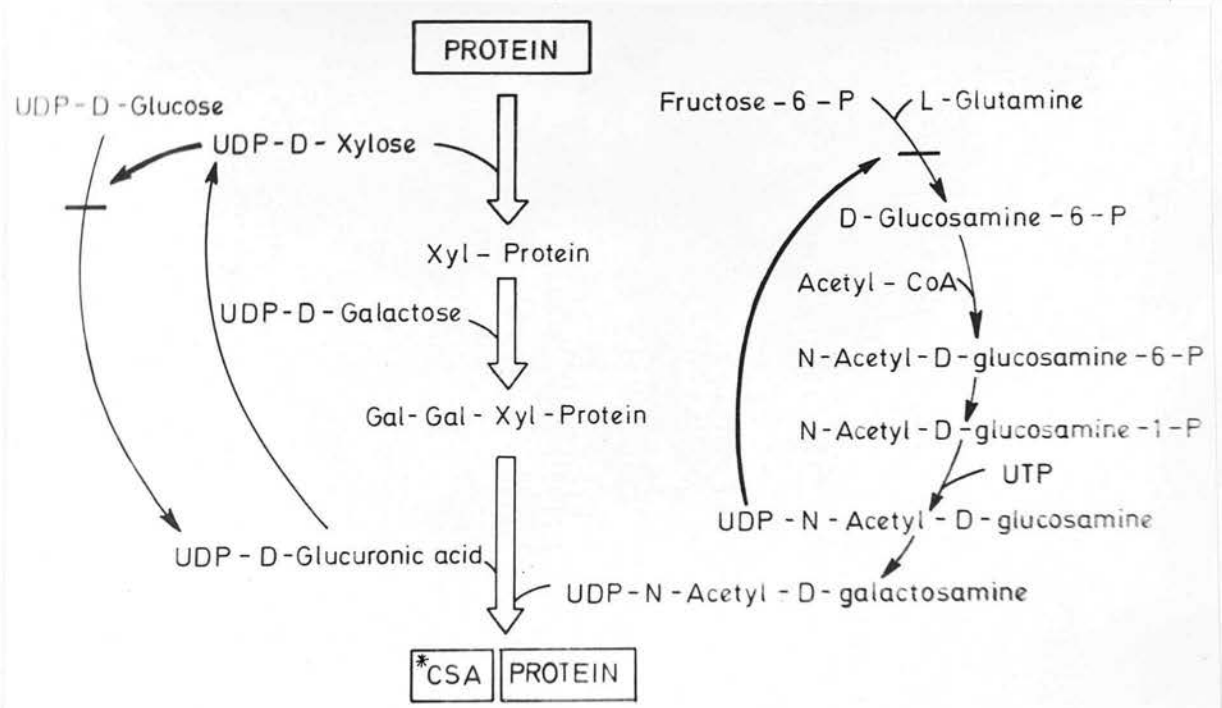



Fig. 1. Pathways of biosynthesis of the chrondroitin-4-sulphate protein complex. Reactions subjected to feed-back regulation are indicated by heavy arrows. (Roden, 1969).

* Chrondroitin sulphate

L-arabinose in human brain hyaluronic acid (Wardi et al., 1966) may represent the actual point of carbohydrate-protein linkage in the same way as xylose joins the two moieties in the chondroitin sulphate protein complex (Roden, 1968).

L-fucose is one of the commonest sugars of the carbohydrate portion of mammalian proteoglycans glycoprotein. Fucose and sialic acid are always peripherally and glycosidically located in the carbohydrate side-chain of the macromolecules with the subterminal sugar, D-galactose. Bray et al. (1946) first isolated L-fucose from a polysaccharide of blood group substances; this sugar has been found in all preparations from A, B, H and $Le^a$ (cf. Kent, 1967; Table 7) and it is unique in that the L-fucopyranosyl residues contribute to the specificity-determining structures of the H, $Le^a$ and $Le^b$ blood group substances (Watkins, 1966).

Further findings of xylose, arabinose and fucose in mammalian macromolecules are given in Table 2 which may be far from complete.

TABLE 2

Findings on the presence (+) of xylose, arabinose and fucose in mammalian mucosubstances

| TISSUE | Ara | Xyl | Fuc | References |
|---|---|---|---|---|
| HUMAN: | | | | |
| Brain | | | | |
| Chondroitin 4-sulphate | | + | | Wardi et al. (1966) |

| TISSUE | Ara | Xyl | Fuc | References |
|---|---|---|---|---|
| Hyaluronate | + | | | Wardi et al. (1966) |
| Aorta | | | | |
| Heparin sulphate | | + | | Knecht et al. (1967) |
| Plasma | | | | |
| Orosomucoid | | | + | Kent (1962 & 1967) |
| | | | | Dische (1963) Jeanloz (1964) |
| Albumin | | | + | Winzler (1958) |
| $\alpha$, $\beta$ & $\gamma$ globulin | | | + | Winzler (1958) |
| | | | | Dische (1963) |
| IgG | | | + | Rosevear & Smith (1961) |
| | | | | Clamp & Putman (1964) |
| Homogeneous globulin prepn. of haptoglobin I, $\alpha_2$, 19S glycoprotein, Ba $\alpha_2$ glycoprotein, Transferrin, Ceruloplasmin, 19 S $\gamma$-globulin $\gamma$ II$_{1,2}$— globulin | | | + | Dische (1963) |
| Blood Group Substances | | | | |
| Erythrocytes | | | + | Koscielak (1963) |
| Ovarian cyst fluid | | | + | Morgan (1962) |
| | | | | Watkins (1966) |

| TISSUES | Ara | Xyl | Fuc | References |
|---|---|---|---|---|
| Erythrocyte stroma of new born infant | | | + | D. 'Elia & Zacchello (1968) |
| Chorionic gonadotrophin | | | + | Bahl (1969) |
| Umbilical cord | | | | |
| Chondroitin 6-sulphate | | + | | Helting & Roden (1968) |
| Placenta | | + | + | Tomoda & Murayama (1965) |
| Bile | | + | | Weicker & Grasslin (1966) |
| Kidney basement membrane | | | + | Dische et al. (1965) |
| Rib cartilage | | | + | Bray et al. (1967) |
| Salivary secretion | | | + | Caldwell & Pigman (1966) |
| | | | | Mandel & Ellison (1962) |
| Gastric secretion | | | + | Schrager & Oates (1968) |
| Intestinal secretion | | | + | Pigman & Teltamanti (1968) |
| Cervical mucus | | | + | Gibbons & Roberts (1963) |
| Sweat | | | + | Pallavicini et al. (1962) |
| Milk oligosaccharides | | | + | Malpress & Hytten (1958) |
| | | | | Kuhn (1958) |
| | | | | Montreuil (1960) |
| Urine | | | | |
| Keratosulphate | | | + | Wessler (1971) |
| Glycoprotein | | + | | Weicker & Grasslin (1966) |
| O - Xylosyl-serine | | + | | Tominaga et al. (1965) |
| OTHER MAMMALS | | | | |
| Bovine nasal septa | | | | |

| TISSUES | Ara | Xyl | Fuc | References |
|---|---|---|---|---|
| Chondroitin 4-sulphate | | + | | Gregory et al. (1964) |
| | | | | Roden & Lindahl (1965) |
| Porcine rib cartilage | | | | |
| Chondroitin 4-sulphate | | + | | Katsura & Davidson (1965) |
| Cattle lens capsule | | | + | Dische & Zelmenis (1964) |
| Pig skin | | | | |
| Dermatan sulphate | | + | | Fransson (1968) |
| | | | | Stern (1968) |
| Pig intestine | | | | |
| Heparin | | + | | Lindahl & Roden (1965) |
| | | | | Lindahl (1966) |
| Pig colonic mucin | | | + | Inoue & Yosizawa (1966) |
| Pig submaxillary mucin | | | + | Carlson (1968) |
| Pig liver and muscle autolysate | + | | | Jelenkova et al. (1961) |
| Sheep colonic mucin | | | + | Kent & Marsden (1963) |
| Calf thyroglobulin | | | + | Spiro (1965) |
| Bovine cervical mucus (oestrus) | | | + | Gibbons (1959) |
| Bovine cervical mucus (pregnant) | | | + | Gibbons (1959) |
| Calf tracheal epithelium | | + | + | Kent et al. (1969) |
| Ewe's milk | | | + | Demanur et al. (1958) |
| Dog liver | + | | | Jelenkova et al. (1961) |

II Ribose

The presence of free ribose has been reported in beef, lamb and pork muscle (Macy et al., 1964), in muscle of cattle and in human skin (Jacobi, 1969). More important is the fact that D-ribose is a component of RNA and nucleotides of all living cells. Much work has been done on the mechanism by which it is formed. The distribution of isotope in the nucleic acid ribose isolated from a HeLa cell suggested its synthesis via both oxidative and non-oxidative steps of the hexose monophosphate pathway (HMP), with predominance of the latter (Hiatt, 1957). This study also indicates the direct incorporation of ribose into nucleic acid rather than following conversion to hexoses. The role of dietary ribose (Table 4) in these reactions is not known.

The labelling of glycogen glucose and RNA ribose after administration of (2 $^{14}$C) glucose in rats suggests that ribose may be synthesized from glucose, and pentose conversion to glucose via the transketolase reaction may play a role in the formation of the glucose of glycogen (Marks & Geigelson, 1957). Similar studies with rat, mouse and human tissues in vitro after administration of (2 $^{14}$C) glucose indicate that each of these tissues can synthesize its own ribose (Hiatt & Lareau, 1960). In contrast to the situation in most rat tissues (Hiatt & Lareau, 1960) and in HeLa cells (Hiatt, 1957), ribose synthesized in human tissue both in vivo and in vitro seems to be derived primarily by way of the oxidative reactions of the HMP (Hiatt & Lareau, 1960).

## B. METABOLISM AND EXCRETION OF ALDOPENTOSES IN MAN

### I. Experiments showing that aldopentoses are metabolized by man

a) Sugars given orally:

Grafe & Reinwein (1932) found that, on the average, 60% of D-xylose was utilised by healthy and diabetic humans and dogs. Total blood reducing sugar in both normal men and dogs was raised after oral administration of xylose.

Marble & Strieck (1932) by long and short term experiments in a respiration chamber showed that both normal and diabetic subjects could oxidise xylose since there was a distinct rise in respiratory quotient after it's administration even to severe diabetics, as well as in depancreatised and phloridzinized dogs. In diabetics the reason of this rise is not well understood, for if xylose contributes to the rise in "ineffective" blood glucose, it should not raise the R.Q. However aldose reductase has a greater affinity for free xylose than for free glucose (Hayman & Kinoshita, 1965) and thus this pentose may be more rapidly introduced, via xylitol, into oxidative catabolism, than is glucose. In addition it is now known that xylitol is rapidly and completely metabolised by man (Lang, 1969).

After oral administration of 25 g of xylose in man, 6 - 7 g was excreted during 5 hours (Fourman, 1948; Brien et al., 1952); the retained portion must have been utilised in some way.

b) Sugars given intravenously:

That xylose is utilised by the human was also shown by Keith et al. (1934), who infused 10 g of xylose into two subjects and recovered 57% in the urine. The plasma xylose and the red cell xylose measured by copper reduction after fermentation were found to be equal.

Wyngaarden et al. (1957) infused D-xylose, D-arabinose, L-arabinose and D-lyxose into healthy fasting subjects and showed that the removal of these pentoses follows a first order of reaction kinetics. Each pentose disappears from the blood at a rate proportional to its concentration and at rates considerably slower than those of the hexoses, D-glucose, D-fructose, D-galactose or D-mannose (Table 3).

TABLE 3

Rates of disappearance from blood and the excretion of pentoses and hexoses in urine.

(Wyngaarden et al. 1957)

| Sugar | Disappearance from blood | Excretion in urine |
|---|---|---|
| | % per min | % of administered dose |
| D-galactose | 6.93 | 10 |
| D-fructose | 3.84 | 4 |
| D-glucose | 3.47 | 2 |
| D-mannose | 1.82 | 45 |
| D-lyxose | 1.14 | 46 |
| D-xylose | 0.98 | 42 |

| Sugar | Disappearance from blood | Excretion in urine |
|---|---|---|
| | % per min | % of administered dose |
| D-arabinose | 0.94 | 51 |
| L-arabinose | 0.96 | 47 |

It is of interest that there is also a correlation between the order of these rates and of the order of rates of absorption of these sugars from the intestinal lumen (Cori, 1925).

Table 3 shows that about 45% of I.V. administered pentoses are excreted unchanged in urine. A few studies with D(1- $^{14}C$) xylose by these authors showed that this sugar is in part rapidly metabolised, for expirated air contains a maximum of $^{14}CO_2$ 45 min after infusion. However the first $CO_2$ sample, collected after 10 min, was already appreciably labeled and $^{14}CO_2$ was still present in detectable amounts for six hours. Half of the 60% of non-excreted pentose was recovered in expired $CO_2$. The other half was presumed to have entered a non-carbohydrate pool and utilised in part for tissue synthesis.

Segal et al. (1957) found that giving insulin, after I.V. infusion of D-xylose, L-arabinose, D-xylose and D-arabinose, lowered abruptly the blood concentrations of the first three sugars. Insulin is known to facilitate entry of a wide variety of sugars into erythrocytes and somatic cells and hence can promote their metabolism.

Segal & Foley (1958) made an extensive metabolic study of

D-ribose on 1 female and 6 males (healthy fasting volunteers) and on three subjects with diabetes mellitus. After I.V. infusion ribose removal from blood was a first order of reaction kinetics. The rate constant of disappearance was calculated to be 3.8 - 5.8% per min after a 20 g dose and 13.8% per min after a 3 g dose; whereas the rate constant of the disappearance for the tracer dose of (1-$^{14}$C)-ribose (5 uCi in 2.5 mg ) was 0.61% per min and for a 20 g plus tracer dose was 1.16% per min. This slow rate constant of $^{14}$C disappearance was due to the simultaneous appearance of non-ribose $^{14}$C compounds (see later).

About one-fifth (21%) of the non-labeled ribose dose appeared in the urine, mostly in 90 min after infusion. The excretion decreased with diminution of the dose. When 20 g of ribose was given at a constant but slow rate only 5 - 12% was excreted. These results contrast with those obtained with D-xylose when the urinary loss was approximately the same irrespective of the dosage or the rate of infusion. Ribose appears to be utilised more completely and more rapidly than D-xylose or L-arabinose especially when time is allowed for its metabolism. When a dose of (1- $^{14}$C)-ribose (5 uCi in 2.5 mg ) was given only one tenth of the administered $^{14}$C appeared in the urine.

The excreted $CO_2$ was labeled 5 min after the end of the 5 min infusion period in both tracer and tracer + load studies. After ribose the total $^{14}CO_2$ in the expired air for 6 hours showed that 48% of the labeled ribose administered had been oxidised.

These studies show that D-(1-$^{14}$C) ribose is metabolised rapidly with prompt excretion of a portion of $^{14}$C-1 as $^{14}CO_2$. Furthermore this $^{14}$C experiment showed that the non-excreted ribose must be largely converted to glucose by the sequence of the HMP from which glucose 6-phosphate can either be oxidised directly, with loss of $CO_2$ to ribulose-5-phosphate or else be degraded by anaerobic glycolysis and the aerobic Krebs' cycle. Since ribose is converted to glucose, it appears probable that the $^{14}CO_2$ in expired air is derived from the oxidation of labeled glucose-6 phosphate at the start of the HMP.

II. Effects of I.V. administered aldopentoses on blood glucose concentrations.

The blood glucose concentration rose by 10 - 32 mg % after I.V. infusion of 5 to 20 g of D-xylose; L-arabinose showed a similar effect. (Wyngaarden et al.,1957). This could be because xylose can eventually join the systemic blood sugar pool and because, in rats at least, kidney cortex can convert some pentoses into glucose (Krebs & Lund, 1966). In contrast to the effect of D-xylose and L-arabinose by I.V. infusion D-ribose lowers the plasma glucose level in man. (Bierman et al.,1959; Segal et al.,1957; Segal & Foley 1958; Steinberg et al.,1967). It was further observed that hypoglycaemia was more prolonged after insulin administration followed by ribose than after insulin alone (Segal & Foley, 1958). The same effect of ribose on plasma glucose has been shown in dogs. (Heteryi & Ishiwata, 1968). It is not well understood how this

happens but mobilization of insulin may be partially responsible for the D-ribose-induced hypoglycaemia. Steinberg et al. (1967) concluded that D-ribose-induced hypoglycaemia has a dose response relationship. Although the concentration of serum insulin was transiently elevated during the infusion of D-ribose, the concentration of insulin attained was probably insufficient in itself to account for the hypoglycaemia. On the other hand the inhibition of phosphoglucomutase by ribose-5-phosphate does not explain satisfactorily the cause (Segal & Foley, 1958).

III Experiments showing that aldopentoses are metabolised by mammalian white blood cells and erythrocytes

Stjernholm & Noble (1961, 1963) showed that $^{14}C$-sugars, including L-arabinose, D-ribose and D-xylose when incubated with rabbit polymorphs gave rise to glycogen and lactate. Stjernholm et al. (1969) found that human lymphocytes metabolise labelled D-xylose and D-ribose. In the above studies the pentoses were metabolised via the transketolase - transaldolase sequence of HMP to fructose-6-phosphate and subsequently by glycolysis. D-ribose is metabolised by human erythrocytes (Bucols & Bartlett, 1960; Lachhein & Matthies, 1960; Lachhein et al., 1961) in contrast to the inability of erythrocyte to catabolise D-xylose and L-arabinose (Lachhein & Matthies, 1960).

IV Mammalian enzymic systems which oxidise or reduce aldopentoses

a) Oxidation of aldoses to aldonic acids:

Ox liver glucose dehydrogenase appears to be specific for

the β-anomers of D-glucose but β-D-xylose can also be slowly oxidised (Strecker & Korkes, 1952). This dehydrogenase can oxidise D-xylose, besides glucose and galactose (Brink, 1953); NAD, NADP and deaminated NAD can all serve as hydrogen acceptors. In vitro D-xylose was not found to be phosphorylated by the calf lens but was oxidised to xylonic acid by a dehydrogenase using NAD (van Heyningen, 1958). Penetration of glucose into the intact lens was inhibited by xylose.

Mammalian aldose dehydrogenase (Cuatrecasas & Segal, 1966) preferentially oxidises D-galactose (100%) but also oxidises L-fucose (3%), D-ribose (10%), D-xylose (84%) and D-lyxose (22%) to the corresponding aldonic acids. Schiwara et al. (1968) separated glucose, galactose and pentose dehydrogenases from pig liver. The pentose dehydrogenase, which is NADP dependent, oxidises D-ribose, D-xylose, L-arabinose, D-galactose and D-glucose in order of preference to the corresponding aldonic acids. Such acids are probably lost to the normal pathways of carbohydrate metabolism unless they undergo transformations similar to those by which L-gulonic acid is an intermediate between D-glucuronic acid and L-ascorbic acid. (Burns & Evans, 1956).

b) Reduction of aldoses to alditols:

Aldose reductases which convert numerous aldoses to their related alditols appear to be widespread. Many such alditols have been detected in normal urine (Pitkanen et al.,1964). Few actual tissues have been studied in respect of this activity, however.

Calf lens aldose reductase (dependent on NADP.2H) reduces a wide variety of aldoses and aldehydes including glucuronolactone and glucuronic acid (Hayman & Kinoshita, 1965). In vitro, this enzyme has greater affinity for D-xylose than for D-glucose. It is stimulated by $SO_4^{2-}$ and is similar to, but not identical with the enzymes of seminal vesicles and placenta (Hers, 1956). In vitro, the intact rat lens can reduce D-xylose to xylitol using NADP.2H. This xylitol can be reoxidised by the lens to form both D-xylose and D-xylulose in the presence of NADP and NAD respectively (van Heyningen, 1959).

c) Oxidation of alditols to uloses and reduction of uloses to alditols:

The alditol oxido-reductase (L-iditol dehydrogenase) of sheep liver can oxidise L-arabinitol to probably 95% of L-ribulose and 5% of L-xylulose (Smith, 1962). By failure of such a mechanism the L-arabinitol isolated by Touster & Harwell (1958) from L-xylulosuric urine could have arisen. L-xylulose in "essential pentosuria" is probably due to deficiency in NADP-xylitol (L-xylulose) dehydrogenase in the liver when some of the accumulated L-xylulose might be slowly reduced to L-arabinitol by alditol reductase (Touster, 1959). Smith (1962) argued this hypothesis because if the L-iditol dehydrogenase of human liver has the same specificity as that of sheep liver, then it may not be able to reduce L-xylulose to L-arabinitol.

However the isolation of L-arabinitol from xylulosuric urine,

and the presence of appreciable quantities of arabinitol in urine of two normal subjects after administration of D-glucuronolactone (Touster & Harwell, 1958) suggest that L-arabinitol is a metabolic intermediate in mammalian system.

Guinea pig liver mitochondria contain two enzymes which make possible the interconversion of L-xylulose and D-xylulose. (Hollman & Touster, 1956). The NADP-dependent enzyme dehydrogenates xylitol to L-xylulose. The NAD-dependent enzyme catalyses the oxidation and reduction of xylitol and D-xylulose respectively. Touster et al. (1956) showed that the above liver enzyme reduced L-xylulose to xylitol. Reversibility of the reaction was indicated by formation of ketopentoses from xylitol. Hollman & Touster (1957) showed that an alditol oxidoreductase from guinea pig liver mitochondria and NADP selectively catalyse the interconversion, xylitol$\rightleftharpoons$L-xylulose. The NAD dependent oxidoreductase catalyses, beside others, the interconversion of xylitol and D-xylulose.

D-ribitol can be oxidised by an alditol oxidoreductase to D-ribulose (Wood et al., 1961) which is further metabolised after phosphorylation. An NAD-dependent oxidoreductase may also interconvert ribitol and D-ribulose (Hollman & Touster, 1957).

## V Metabolic pathways involving the aldopentoses in mammalian systems

The utilisation and excretion of these sugars raise

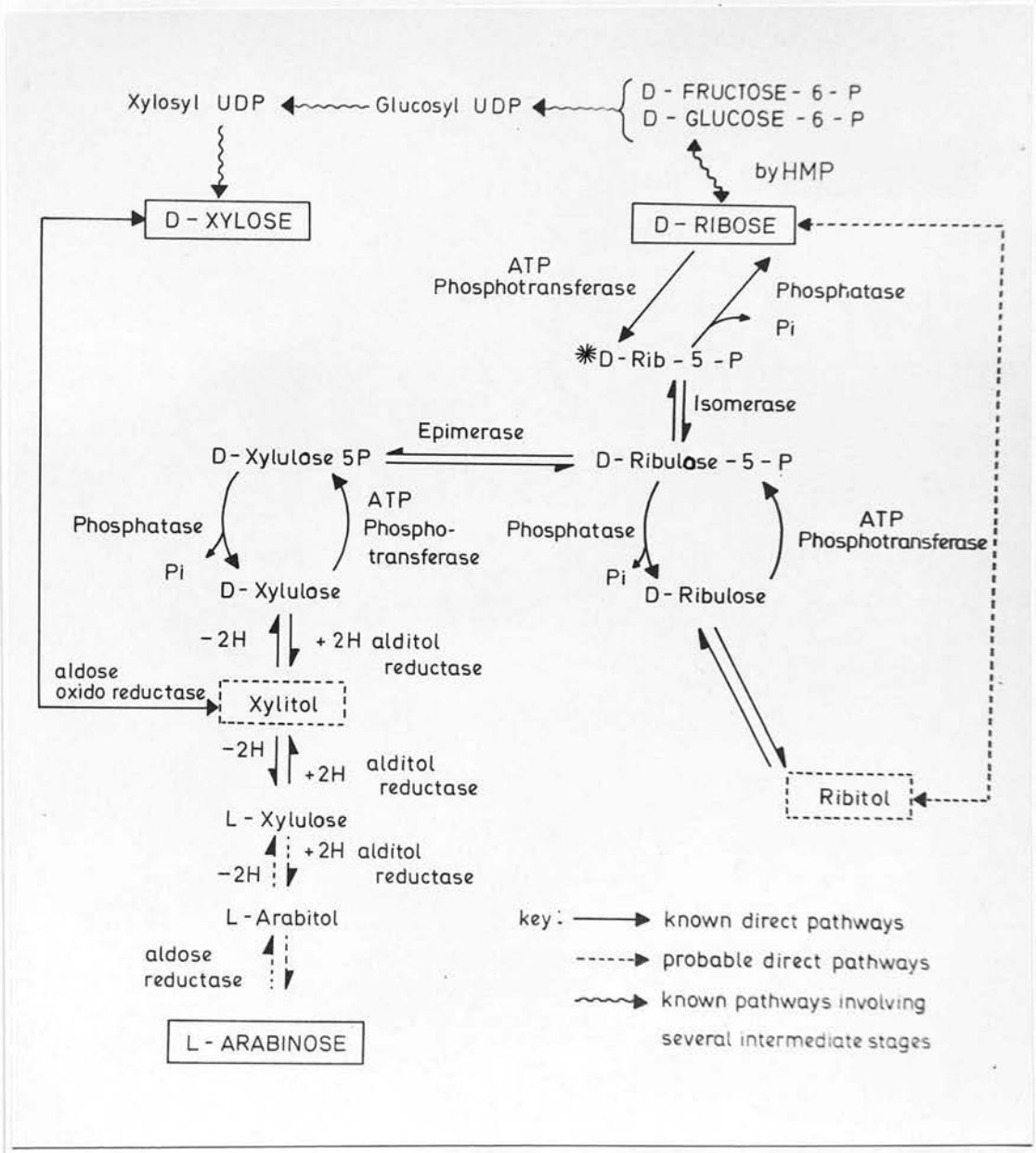



Fig. 2. A diagramatic scheme showing metabolic inter-relationships between the free aldopentoses, the alditols and the pentuloses.

* D-Ribose-5-P.

questions regarding their involvement in the metabolic pathways. The diagramatic scheme in fig. 2 outlines relationships between the free aldopentoses, the alditols and the pentuloses.

The possibility of early phosphorylation of the aldopentoses in the metabolic pathway is suggested by the observation that serum inorganic phosphate falls during I.V. pentose infusion (Wyngaarden et al., 1957; Segal & Foley, 1958). The only mammalian pentose kinase so far isolated and characterised is D-ribokinase from calf liver which catalyses phosphorylation of D-ribose to the 5-phosphate in presence of $Mg^{2+}$ and ATP (Agranoff & Brady, 1956) thus permitting the sugar to enter the HMP. D-xylulose is phosphorylated in the calf liver to form xylulose 5-phosphate. (Hickman & Ashwell, 1956). The reactions of HMP and its link with the glycolytic pathway are outlined in fig. 3, but it is generally accepted that many non-specific phosphokinase reactions take place involving natural and unnatural sugars.

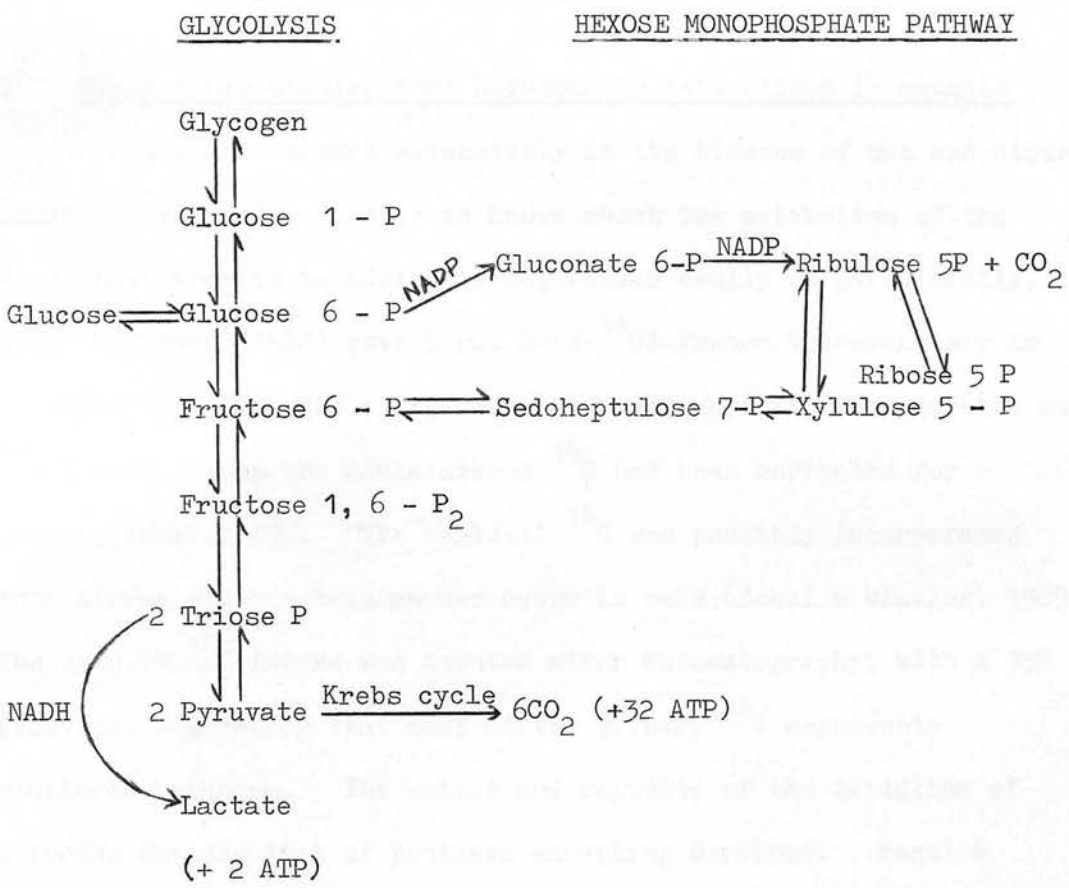



Fig. 3 Pathways of carbohydrate metabolism

(slightly modified from Horecker, 1969).

## C. FUCOSE IN MAMMALIAN METABOLISM

### I Experiments showing that L-fucose is metabolised in mammals

L-Fucose occurs extensively in the tissues of man and other mammals (Table 2). Little is known about the metabolism of the free sugar when it is administered, either orally or parenterally. Segal & Topper (1960) gave 5 uCi L-(1-$^{14}$C)-fucose intravenously to a healthy adult. 56% of the injected radioactivity was excreted as $^{14}CO_2$ in 6 h when the administered $^{14}$C had been corrected for urinary loss of 30%. The residual $^{14}$C was possibly incorporated into tissue glycoprotein as can occur in rats (Bocci & Winzler, 1969). The urinary $^{14}$C fucose was counted after chromatography, with a 93% recovery, suggesting that most of the urinary $^{14}$C represents unaltered L-fucose. The extent and rapidity of the oxidation of L-fucose exceeds that of pentoses excepting D-ribose. Segal & Topper (1960) quoted their unpublished observations of extensive oxidation of L-fucose in the intact rabbit and in liver and kidney slices therefrom.

Kidney homogenates from rats, pigs and cattle caused L-fucose to disappear in the presence of ATP and $Mg^{2+}$. (Kositkovskya & Rosenfeld, 1962) and these authors considered that the first product was fuculose.

Kent & Mah (1961) showed that the mucosal cells from the sheep's colon contained soluble substances containing fucose. This mucosa, after homogenization at pH 7.6 and high-speed centrifugation gave a

soluble enzyme system which on incubation with L-fucose, ATP and $Mg^{2+}$ resulted in the removal of the sugar. The product of the reaction formed a barium salt and was therefore a fucose phosphate. From the works of Ishihara et al. (1968) it can be assumed that L-fucose, if formed, was utilised through GDP-fucose (see later).

In contrast with the findings in man of Segal & Topper (1960) the intraperitoneal (Coffey et al., 1964) and the oral and parenteral administration (Bocci & Winzler, 1969) of L- (1- $^{14}C$)-fucose to rats was followed by almost no utilisation. The $^{14}CO_2$ found after the oral dose was due to oxidation by the intestinal microflora. (Bocci & Winzler, 1969).

II Metabolic pathway and biosynthesis of fucose:

Suggestions regarding the metabolic pathway and biosynthesis of L-fucose in mammalian systems have only been put forward recently.

a) Fucose synthesis through guanosine diphosphate (GDP) - Mannose:

Segal and Topper (1960) attempted to delineate in man the relationship between D-glucose and L-fucose by studying the formation of the latter in human milk. D-(6- $^{14}C$) glucose (5 uCi) were infused intravenously to each of two lactating women. Because both fucose and glucose moieties in the milk were similarly labelled, they suggested that, like certain micro-organisms (see below), man synthesises L-fucose predominantly by direct conversion from

D-glucose without rupture of the carbon chain.

Isotope experiments *in vivo* with several micro-organisms (Segal & Topper, 1957; Wilkinson, 1957; Heath & Roseman, 1958) indicated that the carbon chain of D-glucose remained intact during the multistep enzymatic conversion to L-fucose. The labelling pattern of L-fucose was essentially the same as in D-glucose.

The sugar nucleotide, GDP-fucose was isolated from a strain of *Aerobactor aerogenes* (Ginsburg & Kirkman, 1958). This sugar nucleotide could be formed from GDP-mannose by dialysed crude extracts of *A. aerogenes* in the presence of NADPH (Ginsburg, 1958). Further, Ginsburg (1960, 1961) isolated an enzyme from *A. aerogenes* which transformed GDP-D-mannose in presence of NADPH and $NAD^+$ into GDP-L-fucose and showed that guanosine 5 - diphosphate 6-deoxy-4-oxo-D-mannose was an intermediate.

The identification of GDP-fucose in sheep's milk (Demanur *et al.*, 1958) and GDP-mannose in bovine mammary gland (Carlson & Hansen, 1961) suggested that a system similar to that in *Aerobactor* might be responsible for the mammalian biosynthesis of L-fucose. Foster & Ginsburg, (1961) indeed found such evidence. They used rabbit lung, intestine and other tissues which were capable of forming GDP-fucose from GDP-mannose. Segal & Topper's (1960) finding strongly suggests the possibility of a similar mechanism of fucose biosynthesis in the human.

b) Evidence of direct "activation" of fucose and its synthesis through GDP-fucose:

In Esch. coli the established pathway for the introduction of L-fucose into the glycolytic pool involves the following reactions:

a) L-fucose $\rightleftharpoons$ L-fuculose (Green & Cohen, 1956; Huang & Miller, 1958; Heath and Ghalamber, 1962).

b) L-fuculose + ATP + $Mg^{2+}$ $\rightleftharpoons$ L-fuculose + P + ADP (Huang & Miller, 1958; Heath & Ghalamber, 1962).

c) L-fuculose-1-P $\rightleftharpoons$ L-lactaldehyde + dihydroxyacetone phosphate (Ghalamber & Heath 1962).

In studies on the metabolism of L-fucose in the rat Coffey et al. (1964) found after administration of $^{14}C$ fucose that the intestinal acidic glycoprotein contained the label only of fucose but not of mannose which should have also been labelled if fucose synthesis occurred through GDP-mannose. Alternate pathways of fucose metabolism in mammalian tissue became known after the work of Ishihara et al. (1968). They isolated ATP-L-fucose kinase from pig liver which phosphorylated the sugar at C-1 in presence of $Mg^{2+}$. Ishihara & Heath (1968) also isolated a second enzyme, guanosine diphosphate L-fucose pyrophosphorylase which catalysed the reaction $\beta$-L-fucose 1-phosphate + guanosine triphosphate $\rightleftharpoons$ GDP-L-fucose + $PP_i$. This is the first direct evidence for a mammalian enzyme system which would permit the direct utilisation of exogenous L-fucose. L-($^{14}C$) - fucose is directly incorporated into macromolecules of HeLa

cells (Kaufman & Ginsburg, 1968) with no detectable radioactivity in other component sugars. This observation and the subsequent accumulation of GDP-L-fucose in HeLa cells substantiate the findings of Ishihara et al. (1968). In experiments of Segal & Topper (1960) the oxidation of the sugar to $CO_2$ after L-(1- $^{14}C$)-fucose administration suggests that the utilisation of this sugar in man can take place directly via L-fucose ——→ L-fucose-1-phosphate ——→ L-lactaldehyde and dihydroxyacetone phosphate.

The diagrammatic scheme of the two pathways of the biosynthesis of L-fucose is outlined in fig. 4.

III L-fucose dehydrogenase:

This $NAD^+$-dependent enzyme isolated from pig liver (Schachter et al., 1969) preferentially oxidises L-fucose to L-fuconolactone which spontaneously hydrolyses to L-fuconate. L-galactose and D-arabinose are also oxidised. The metabolic role of this enzyme remains to be determined.

IV Transfucosylation processes:

Transfucosylations involve GDP-L-fucose and fucosyl-transferases which are usually particulate. Such fucosylations of milk saccharides occur in mammary tissues in the bitch (Grollman et al., 1965) and in lactating women (Shen et al., 1968; Grollman et al., 1969).

The blood-group 'H' substance can receive its L-fucose from GDP-L-fucose through catalysis by a particulate preparation from pig

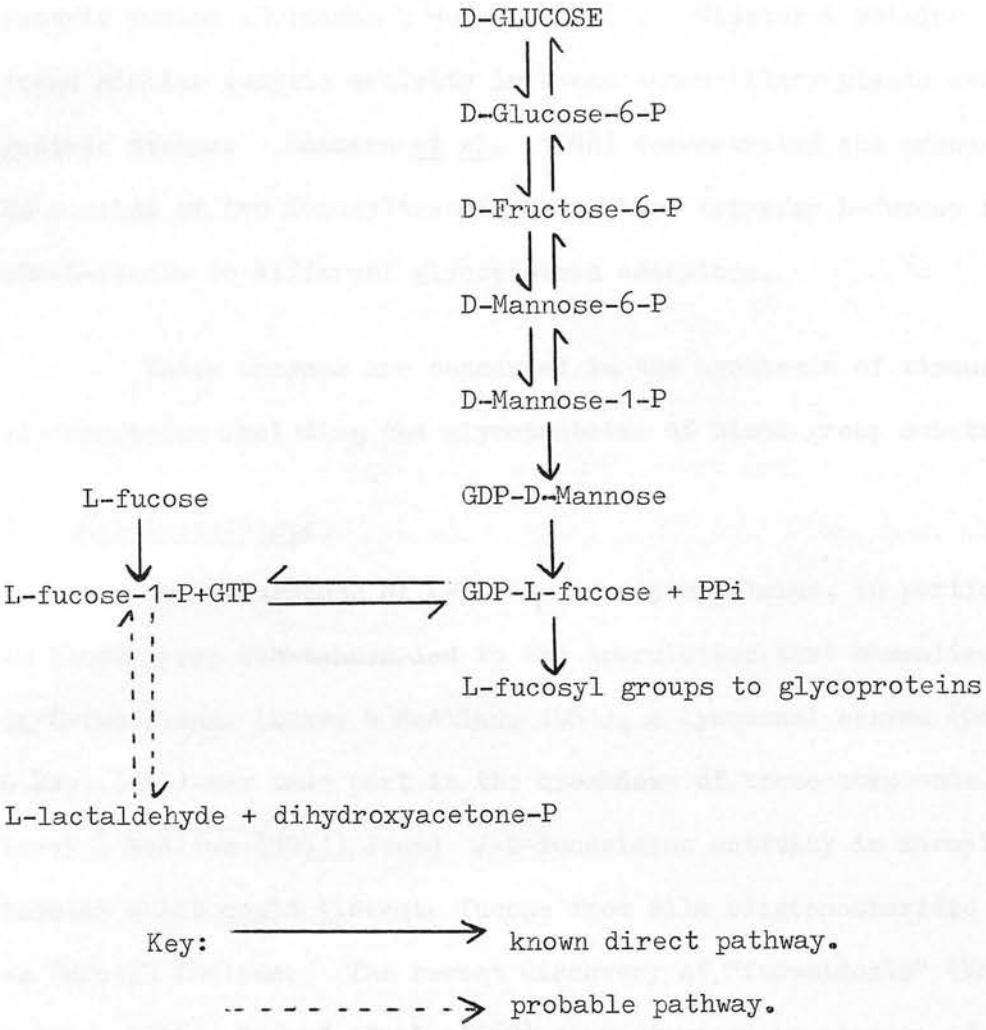



fig. 4 Diagrammatic scheme of the pathways of L-fucose biosynthesis.

gastric mucosa (Grollman & Marcus, 1966). Chester & Watkins (1969) found similar enzymic activity in human submaxillary glands and gastric mucosa. Bosmann et al. (1968) demonstrated the presence in HeLa cells of two fucosyltransferases which transfer L-fucose from GDP-L-fucose to different glycoprotein acceptors.

These enzymes are concerned in the synthesis of tissue glycoproteins including the glycoproteins of blood group substances.

V $\alpha$-L-fucosidase:

The occurrence of L-fucose in glycoproteins, in particular in blood group substances, led to the speculation that mammalian $\alpha$-L-fucosidase (Levvy & McAllan, 1961), a lysosonal enzyme (Conchie & Hay, 1963) may take part in the breakdown of these compounds. Levvy & McAllan (1961) found $\alpha$-L-fucosidase activity in mammalian tissues which could liberate fucose from milk oligosaccharides such as fucosyl lactose. The recent discovery of "fucosidosis" (Van Hoof & Hers, 1968; Durand et al., 1969) characterised by storage of large quantities of fucose-rich glycoproteins with a simultaneous absence of $\alpha$-L-fucosidase activity in different tissues supports the possible "catabolic" role of $\alpha$-L-fucosidase in normal healthy tissue. Vidershayn & Rosenfeld (1969a) showed that pig kidney $\alpha$-L-fucosidase splits fucose from previously fragmented blood group substance A and H. L-fuconolactone acted as a specific inhibitor of $\alpha$-L-fucosidase (Vidershayn & Rosenfeld, 1969b).

Judging from the amount of radioactivity in $CO_2$, urine,

plasma and in the carcass of rats, after administration of labelled L-fucose glycoprotein, it appears that the gastrointestinal tract contains $\alpha$-L-fucosidase which rapidly cleaves the fucose from the oligosaccharide moieties of glycoprotein (Bocci & Winzler, 1969).

## D. AVAILABILITY OF ALDOPENTOSES AND FUCOSE TO THE HUMAN BODY FROM EXOGENOUS SOURCES

Whatever the rates and mechanisms involved in the intestinal absorption of aldopentoses and fucose, it is well known that the 'available' dietary carbohydrate is absorbed to the extent of 97% in man (e.g. Davidson & Passmore, 1969). Southgate (1965) showed that human faeces normally contain no free sugars. Hence any amounts of free aldopentoses and fucose consumed in the food are practically all absorbed, the amount of which is impossible to assess with present knowledge.

Nature of utilisation of ingested pentosan has been studied by several workers. Colonic destruction of xylans was first suggested some 60-odd years ago. Selliere (1908, 1909) and Swartz (1910 - 11) produced evidence that in man and in some other mammals the enteric flora possesses xylanase activity.

McCance & Lawrence (1929) found that large quantities (30 - 80%) of pentosans disappear from the alimentary canal. (Pentosans are defined as hemicelluloses and are 'unavailable' polysaccharides). They administered pentosan either "pure" or in the form of some natural food and then determined the percentage

excreted in the faeces, the difference representing the amount digested. McCance & Lawrence (1929) ruled out the possibility of hydrolysis of pentosans by hydrochloric acid in the stomach and by the enzymes of the alimentary canal. They suggested that the disappearance of pentosans was due to the action of micro-organisms in the large intestine. The products formed and absorbed are not sugars but fatty acids, so that these polysaccharides can not be regarded as supplying the host with carbohydrates. Recently, *in vitro*, Southgate (1965) showed that pentosans are utilised by the intestinal flora excreted in human faeces. But, subsequently, no free pentoses was detected by paper chromatography. So, it may be presumed that the free sugars produced by the flora from these polysaccharides are immediately oxidised and hence it is unlikely that free pentoses are absorbed from breakdown of pentosans in the large bowel and thus cannot contribute to the urinary pentoses.

Southgate & Durnin (1970) showed that in humans the apparent digestibility of pentosan was up to 98% and pointed out that any breakdown of pentosan was a result of the activities of the intestinal micro-organisms. Whether dietary pentosan e.g. arabinose (with furanosyl linkages) which is easily hydrolysed by very dilute acid *in vitro*, (Aspinall *et al.*, 1967) is split in the human stomach is not yet known. From present knowledge it can be said that pentosans are not as such calorigenic to human and only free xylose and arabinose in food are metabolised in the body.

In a review Bell (1962 ) gave an account of the occurrence

of some commoner monosaccharides including xylose, arabinose, and fucose in free form and in glycosidic combination. McCance & Lawrence (1929) analysed 82 plant foods for pentoses after acid hydrolysis by the method of McCance (1926) which used hydrochloric acid (thus forming furfural which reacted with benzidine). All the fruits, nuts and vegetables analysed contained varying amounts of pentosans, e.g. the pentose content of apples was 15% and scots kale 72% of the total reducing sugar found on hydrolysis. This study, however, does not show how much of these sugars are originally free and how much combined. On hydrolysis, cereals (Preece & Hobkirk, 1953) and soyabean polysaccharides (Aspinall et al., 1967) yield free xylose and arabinose.

Studies on the occurrence of free pentoses in different food stuffs have been limited and perhaps due to use of different methodological techniques sugar contents of the same food were found to vary with different workers. For example, Partridge (1948) by paper chromatography found a sample of apple juice to contain glucose, fructose and sucrose. I have found free xylose and arabinose in apple juice (page 160).

Ribose forms (perhaps a negligibly small) part of the daily carbohydrate intake in man. It is ingested chiefly as the ribonucleic acids of cells.

The following table, which may not be complete, shows the occurrence of free aldopentoses in a number of foods analysed.

TABLE 4

Presence (+) of free aldopentoses in certain items of food

| Foods | Ara | Xyl | Rib | Authors |
|---|---|---|---|---|
| Fish (post mortem) | | | + | Tarr (1955) |
| Codling muscle (chill-stored) | | | + | Jones (1958) |
| Beef and Veal | | | + | Tarr (1955) |
| | | | | Grau et al. (1960) |
| Beef and pork | | | + | Fredholm (1960) |
| Chicken muscle (post mortem) | + | + | + | Lilyblade & Peterson (1962) |
| Beef, lamb and pork | | | + | Macy et al. (1964) |
| Egg (white and yolk) | + | + | + | Tunmann & Silberzahn (1961) |
| Maize flakes | + | | | Macleod & Preece (1953) |
| Apple juice | + | + | + | Letzig & Nuernberger (1963) |
| Grape and raisin juices | + | + | | Ninnis & Ninnis (1957) |
| Mango fruit | | + | | Sarker (1963) |
| Rhubarb | | + | | Chumbalov et al. (1966) |
| Beer | + | + | + | Charles (1962) |
| | | | | Aso et al. (1961) |
| | | | | Hay & Smith (1962) |
| Cognac and plum brandy | + | + | | Lichev & Panaiotov (1958) |
| Wine | + | + | + | Charles (1962) |
| | | | | Esan & Amerine (1966) |

Fucose has not been reported to occur free in any food. Ishihara et al. (1968) commented that L-fucose occurs widely as a component of polysaccharides and of glycoprotein among various plants and animal species so that significant amount of this sugar is consumed in the diets of mammals, which after liberation by intestinal L-fucosidase could be available to the body. Milk oligosaccharides (Kuhn, 1958; Malpras & Hytten, 1958; Montreuil, 1960), broad bean hemicellulose (Kawamura & Narasaki, 1959), soya bean polysaccharide (Kawamura & Narasaki, 1961; Aspinall et al., 1967) and black-bean glycoprotein (Jaffee, 1962) have been reported to contain fucose.

# CHAPTER 3

# THE DEVELOPMENT OF NEW METHODS OF ANALYSIS OF ALDOPENTOSES AND FUCOSE BASED ON THIN-LAYER CHROMATOGRAPHY

# THE DEVELOPMENT OF NEW METHODS OF ANALYSIS OF ALDOPENTOSES AND FUCOSE BASED ON THIN-LAYER CHROMATOGRAPHY

At the start of the present investigation paper chromatography was attempted to separate aldopentoses. After a few unsuccessful attempts and knowing that this procedure is slow, thin-layer systems were examined and proved suitable to separate and, for the first time, to measure aldopentoses and, as well, fucose. Anyone experienced in developing an analytical chromatographic procedure will realise that this involves many trials and a large number of errors. In the following pages only successful systems are described; unsuccessful ones are given in appendix II.

## Glassware

Glass apparatus was cleaned by keeping in pyroneg solution for a minimum of 24 h (4 tablespoonful mixed in a container with 4 gallons of water). Each item was rinsed at least 10 times with tap water and then at least 6 times with glass distilled water and then allowed to dry at room temperature.

## Water

Glass distilled water (GDW) was used in the preparation of aqueous solutions, in the final rinsing of cleaned glassware and during experimental procedures.

## De-greasing solvent

This consisted in 1.5 parts of glass-distilled methanol and

2 parts of chloroform. It was used for drying pipettes and to ensure that chromatographic glass plates were grease free.

Equipments for preparation of t.l.c.

The equipments used were those supplied by Quickfit.

Plate holder frame:

This holds either five 20 x 20 cm or ten 20 x 10 cm or three 20 x 35 cm glass carrier plates. The frame was placed on a level bench and the plates lie adjacent to each other, which were forced against the underside of the side members of the frame by a locking device and thus presented a plane upper surface.

Spreader:

On the sides of the spreader the layer thickness are marked in 0.25, 0.5, 0.75 and 1 mm. The spreader was placed on the first plate of either end with the chosen figure for layer thickness on the lower trailing side. Prepared adsorbent was poured into the spreader, which was then moved at a uniform rate with slight downward pressure until it reached the plate at the other end.

Cleaning of glass carrier plates

These were left in pyroneg water for at least 24 h and then rinsed thoroughly with tap water and GDW. They were dried in a vertical position in an oven at $80^{\circ}$. Each plate was made grease free by wiping it repeatedly with a cleaned glass linen soaked in de-greasing solvent. The plate was wiped dry with another glass linen and placed on the plate holder frame preparatory to spreading the adsorbent.

Preparation of buffered Kieselguhr-G thin-layers

The amounts stated were sufficient to coat 5 plates 20 x 20 cm.

(1) Kieselguhr-G buffered with sodium acetate (acetate plates): 40 g Kieselguhr-G (1 part) (Merck) was blended for 60 s with 120 ml of 0.03 M sodium acetate (3 parts).

(2) Kieselguhr-G buffered with sodium dihydrogen orthophosphate (phosphate plates): 40 g Kieselguhr-G (1 part) (Merck) was blended for 90 s with 100 ml of 0.15 M sodium dihydrogen orthophosphate (2.5 parts).

Slurries were made separately in a blender at low speed. The glass container was shaken gently to aid expulsion of air bubbles from the suspension. The slurry was then spread on the plates to give a thickness of 0.5 mm. After $\frac{1}{2}$ hour the neighbouring plates were separated from one another and allowed to dry at room temperature (between 18-20°C) for 24 h. The plates were stored at the humidity and temperature of the laboratory in a multiple-drawer cabinet. Acetate plates were indefinitely stable in the above conditions and even at 23°. However, if the storage temperature of the phosphate plates exceeded 20° the plates became useless. Both acetate and phosphate plates were normally used within a week of preparation.

Cleaning edge of thin-layer plates and marking the layer

The margins of the thin-layer plates were often not uniform due

to the thickness of the spreader side edge. Before use, the side-margins (2 to 3 mm) of the coating of each plate were removed uniformly by means of a stainless razor-blade. A transverse line (of origin) was drawn with a soft lead pencil at right angles to the length of the plate (i.e. the eventual direction of the solvent flow) at 2 cm above the lower margin. Evenly-spaced pencil spots were made to guide application of samples.

Preparation of stock solutions

The four sugars under study were purified before use. L-arabinose was re-crystallized from aqueous propan-2-ol and D-xylose and D-ribose both from propan-2-ol. Fucose (D- or L-) was re-crystallized from an ethanolic solution kept at $2^{\circ}$. To ensure freedom from pentose, commercial fucose samples were spotted on an acetate plate and stained differentially by the PABA reagent (page 54). One commercial sample did contain some pentose. Rhamnose was re-crystallized from ethyl acetate. Glucose, galactose, fructose and mannose were commercial products.

Stock solutions of these sugars (5 mg/ml) were prepared in water saturated with benzoic acid. These stock solutions kept indefinitely in a stoppered glass container at room temperature.

Preparation of urine for t.l.c.

Preliminary treatment of urine:

All urine samples were measured and timed collections. A volume of about 100 ml was stored at $-20^{\circ}$ in plastic containers with

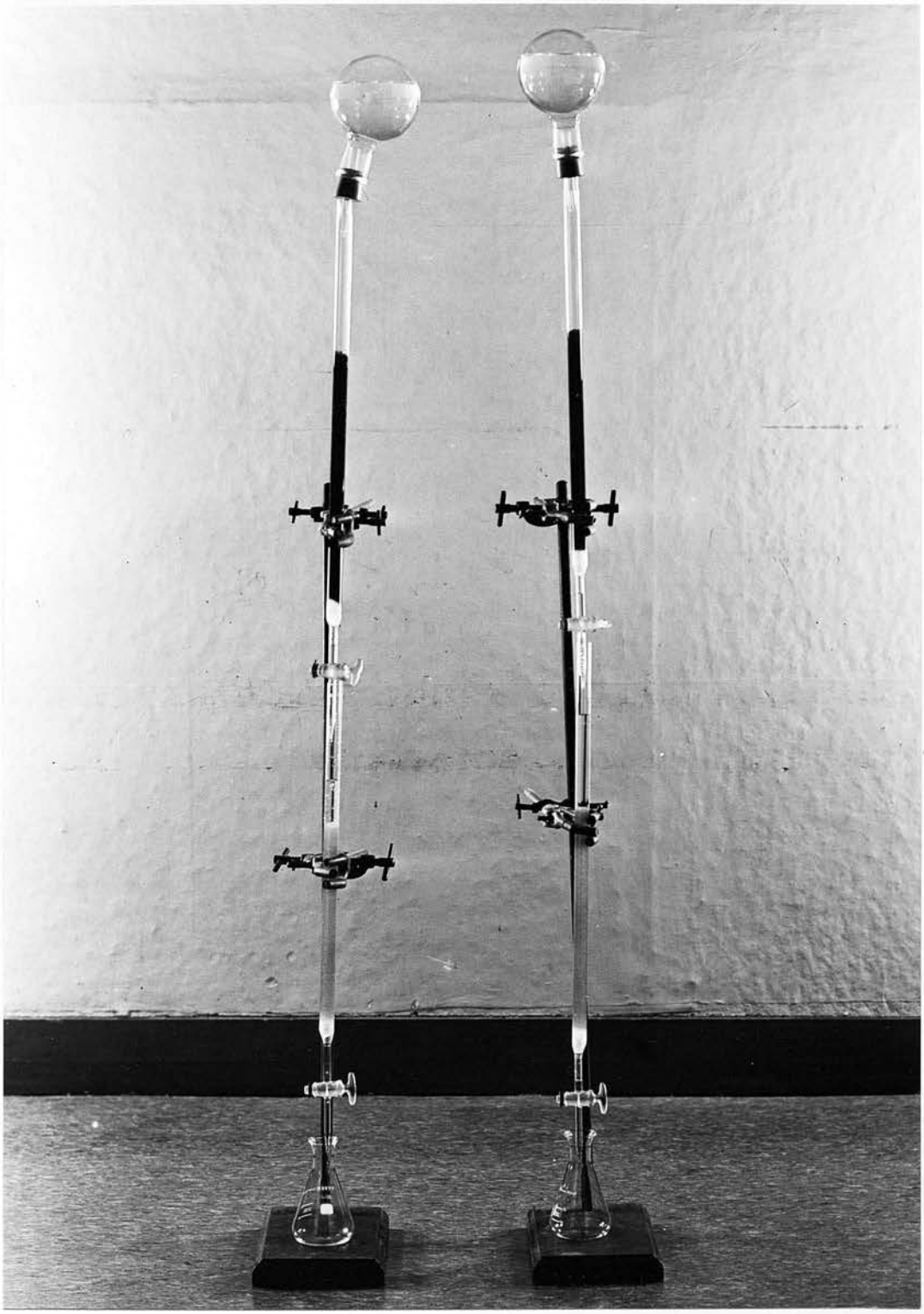


Fig. 5. Glass columns (54 x 1.25 cm) in tandem fitted with burette stop-cocks, used for deionisation of urine.

(Text page 47)

5 ml of toluene if not analysed immediately. Concentrated urines were diluted with GDW to correspond to a excretion rate of 1 ml/min.

Deionisation of urine:

Attempts to remove electrolytes and adsorb sugars directly from urine on to a special charcoal (Hughes & Whelan, 1958) were found to be unsatisfactory at the microgram level (appendix III). However, satisfactory deionisation followed using a modification of that of White & Hess (1956). Two glass columns (54 x 1.25 cm)(page 46 fig. 5) fitted with burette stop-cocks were used in tandem. The upper column contained 15 g of Amberlite IR-120($H^+$) (BDH) and the lower one 15 g of Amberlite IRA-400(acetate) (BDH); the resins were retained by loose plugs of cotton wool. Before introducing urine to the upper column, the flow-rate from each tap was adjusted to give 12 to 16 drops/min (or 0.5 ml/min).

Urine equalling the volume normally excreted in 5 min was introduced to the upper column. A constant level and pressure of water were maintained in the upper column by a dispenser. An eluate of 150 ml was collected; this treatment removed ions including the weak base, urea.

Regeneration of used resins in columns

The either resin (500 g) in a slurry was placed in a glass column (150 x 4 cm) (fig. 6) with a plug of cotton wool at the bottom.

Anionic resin IRA-400 (acetate form):

Amberlite IRA 400 ($Cl^-$) (BDH) was first treated with 5 l of

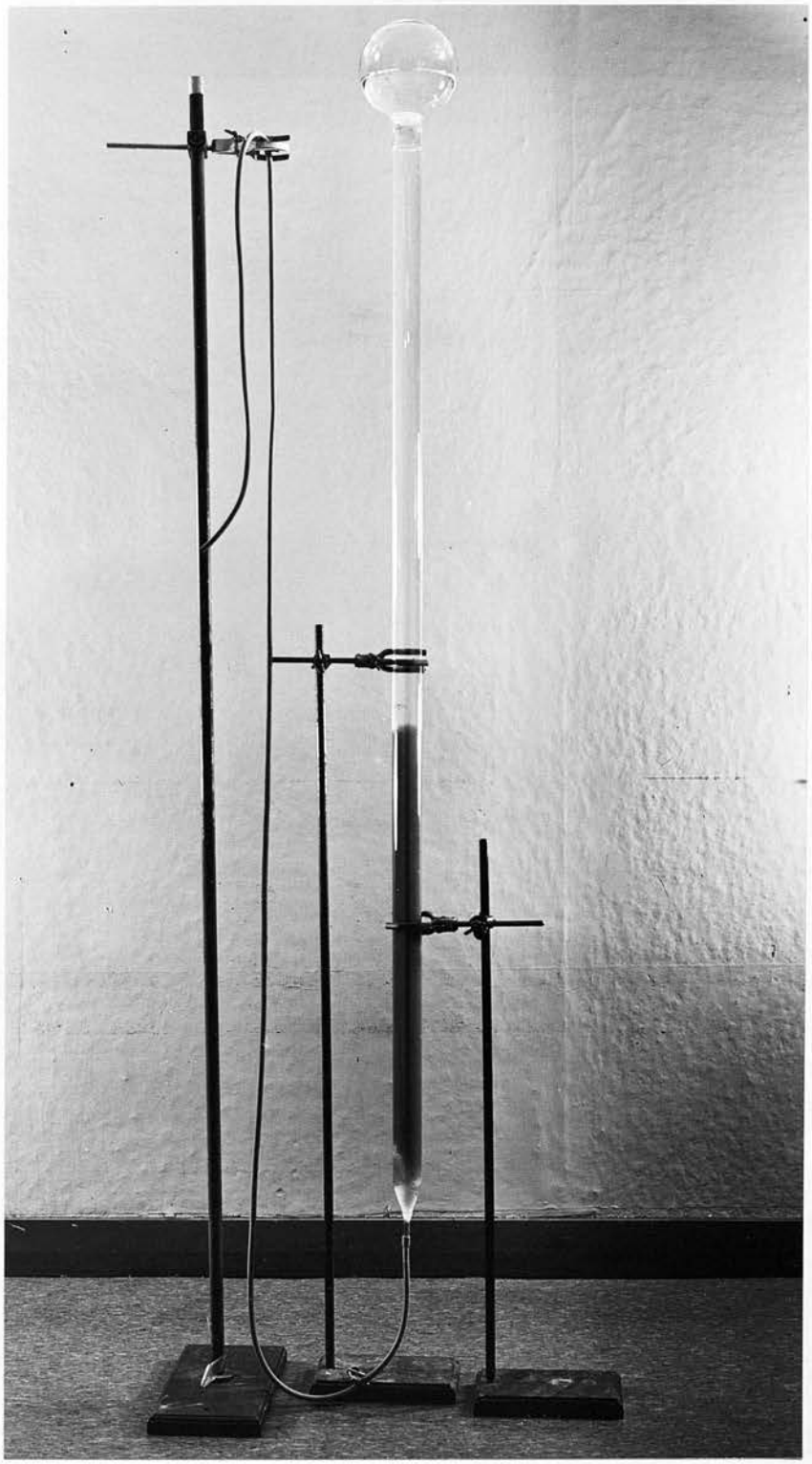


Fig. 6. Glass column (150 x 4 cm) used for regeneration of resins.

4% NaOH solution at a flow rate of 120 ml/min to ensure an 80% conversion to the OH-form (BDH, 1965, Ion exchange Resins, 5th Ed.). Then 40 l of distilled water at the same flow rate was passed through the column, followed by 5 l of 4% acetic acid and, finally 40 l of distilled water. The fluid level in the column was maintained by a dispenser (fig. 6).

Cationic resin IR-120 ($H^+$) (BDH):

This resin was always regenerated before use as follows:- 5 l of 3 N-HCL was passed through 500 g of resin at a flow rate of 70 ml/min (BDH, 1965, Ion exchange Resin, 5th Ed.). At the same flow rate 20 l of water was necessary to wash the resin and obtain a neutral, chloride-free effluent.

Preparation of the urine eluate for t.l.c.

The total eluate (from urine excreted in 5 min) was evaporated to dryness at $45^{\circ}$ in a vacuum rotary evaporator (Stålprodukter, Sweden) (fig. 7). Not more than 50 ml of the eluate was taken at one time in a 250 ml flask to avoid splashing. The residue was then transferred to a 50 ml flask by washing 6 times with 3 ml lots of 80% aqueous methanol and the solvent evaporated completely as before. The resulting residue was carefully dissolved in 0.2 ml of 80% aqueous propan-2-ol (v/v) to give a solution suitable for application to the appropriate thin-layer plates.

Application of standard solutions and urinary concentrate on the thin-layer (t.l.) plates

Acetate plates:

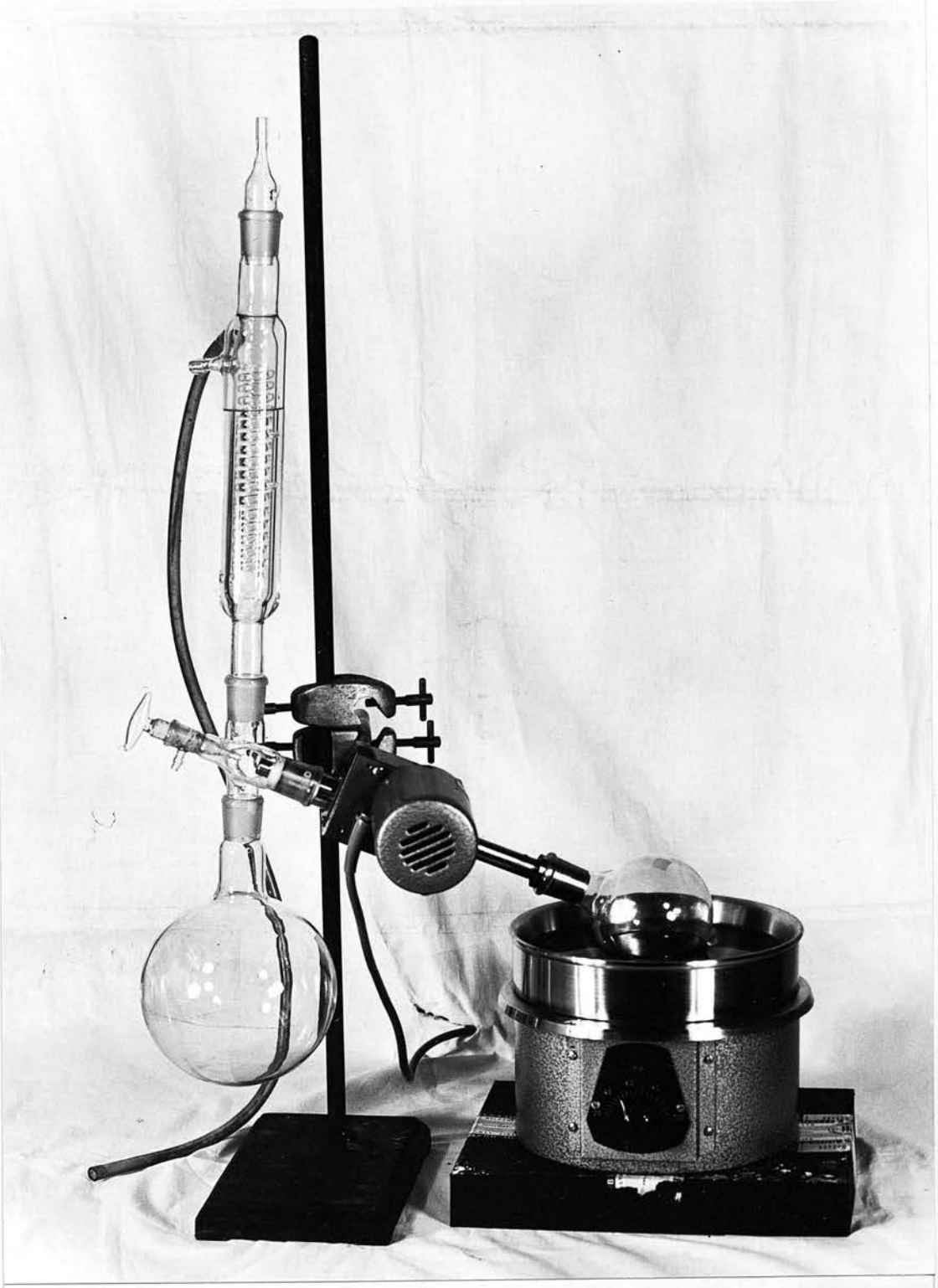


Fig. 7. Rotary vacuum evaporator used for drying the eluates.

On 20 x 20 cm plate 3.0 ul of a standard sugar solution containing arabinose (15 µg), xylose (15 µg) and ribose (15 µg) was applied uniformly on the line of origin in strips about 1.5 cm long from a 5 µl graduated pipette. Besides the above aldopentoses, during preliminary investigations, other monosaccharides such as galactose, glucose, fructose, mannose, fucose and rhamnose were applied to detect the urinary sugars. However, during the presently described experiments only the aldopentoses standards were spotted on acetate plates.

From the final deionised urine concentrate (200 µl) eight 5 µl lots were applied to the same 1.5 cm strip; this volume (40 µl) corresponded to 1 min excretion. The "unknown" strip was placed, horizontally, 3 cm apart from the strip containing the standards. Each plate contained 3 strips; one of standards and two from two urine samples. Duplicate strips on one plate for each sample were not considered necessary in view of the recoveries and reproducibilities (page 8384). In each strip the multiple applications of both unknowns and standards should have ensured good averages.

Between applications of the 5 µl quantities, solvents were dried off in a current of warm air (80 - 84°).

Phosphate plates:

Because, on phosphate plates, the colour developed by the p-anisidine spray (page 56 ) was less intense after extraction of the pigmented spots, it was necessary to apply deionised urine

concentrates in amounts equivalent to 2 min's excretion. In addition, since fucose and xylose were the two sugars which especially separated, only 30 µg amounts of each standard in duplicate, were applied to the plates. This enabled the measurement of fucose, completely separated, and xylose, which although coincident with fucose on acetate plates and with ribose on phosphate plates, could be measured by differences (page 75 ).

Purification of solvents

Solvents of any quality were always purified before use. This was found to be essential since trace impurities, especially carbonyl groups can interfere with quantitative spray colour-developments. All alcohols were distilled over sodium hydroxide and silver oxide. If such treated methanol contained volatile bases, it was redistilled over potassium hydrogen sulphate. Ethyl acetate was distilled after keeping (24 h) over anhydrous $K_2CO_3$. Glacial acetic acid was distilled over potassium permanganate.

Chromatographic development

The tanks were lined with filter paper as a routine but no previous vapour saturation of the atmosphere in the tank with the developing solvent mixture was found to be necessary.

Acetate plates:

The successful developing solvent was slightly modified in its proportions from that used by Stahl & Kaltenbach (1961) and consisted in ethyl acetate-propan-2-ol-water (4:1:0.5). (This mixture remains stable for only 48 h). The t.l. plates were

developed vertically. At room temperature the solvent was allowed to run the full length of the 20 cm plate. This took 55 min. A single development did not separate fructose from arabinose, so, after removing the plates from the tank, the developing solvent was evaporated in a current of cold air for 15 min and a second development done to separate arabinose from the hexulose. The whole operation took 2.5 h.

Phosphate plates:

Two solvent systems gave satisfactory results on 20 cm runs. (1) ethyl acetate-methanol-butan-1-ol-water (16:3:3:2). (2) ethyl acetate-methanol-butan-1-ol-propan-2-ol-water (8:1:1:1:1). (Other successful systems for the separation of fucose are given in appendix I). For the long 35 cm runs (see below) solvent (3) ethyl acetate-methanol-butan-1-ol-water (16:3:3:1) proved useful. All these mixtures were unsuitable after 48 h. Solvents (1) and (2) gave identical results on 20 cm plates when the full length of the plate was run (65 min).

While a single development sufficed qualitatively to separate fucose from all the other sugars, to obtain sufficient spatial separation of fucose as well as all the other individual monosaccharides from each other, except ribose and xylose, two developments were necessary. Single development showed urinary sugars to run slightly behind the corresponding standards. But a second development overcame this. Moreover the sugar spots became more compact and well shaped with a second or third development.

After each run the plates were dried at room temperature for 30 min and then either subsequent development was performed or the plate was sprayed. With double development the whole procedure involved 3 h. Using 20 x 35 cm plates and solvent (3), 3.75 h was needed for a single run.

Visualisation of the sugar spots

Portable fume-cupboard:

A portable fume-cupboard ( 50 x 45 x 38 cm ) with a suitable fan fixed to the back was designed in this laboratory especially for spraying chromatograms (fig. 8).

Spray reagents:

The "Shandon Laboratory Spray Gun" was used. The "dried" plates were placed in the fume-cupboard in a vertical position (fig. 8) and sprayed (from 1.5 ft) uniformly in four directions. Excessive moistening or soaking of the plates with spray reagents was avoided.

A differential qualitative and quantitative spray:

Stannous chloride added (Bell & Talukder, 1971) to the reagent used by Bell (1966) for assay of hexoses and pentose in aqueous solution, stabilised the colours developed on the t.l. chromatograms after heating. The reagent was prepared as follows:- 4-aminobenzoic acid (1 g) (p-aminobenzoic acid) and 0.6 g of 3-Carboxy-4-hydroxybenzene-sulphonic acid (sulphosalicylic acid) each separately dissolved in 45 ml of glacial acetic acid, were added to a solution of $SnCl_2$ (2 g) in 10 ml of water (PABA reagent).

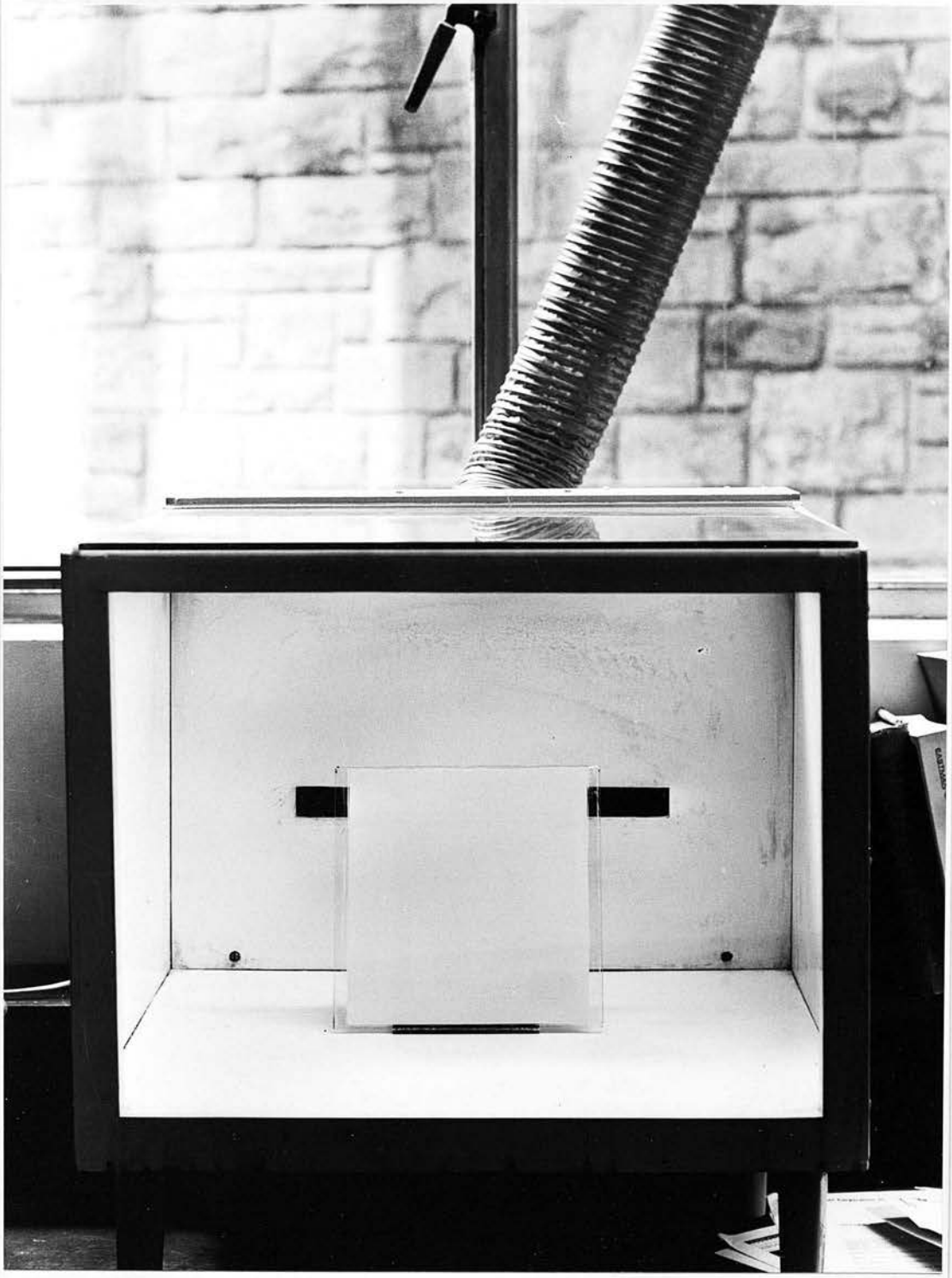


Fig.8 Portable fume-cupboard used especially for spraying thin-layer chromatograms.

The cloudy solution was filtered before use.

Sprayed acetate plates were heated at 100° for 15 min. The colours produced were as follows:- pentoses, cerise; aldohexoses, brown-yellow; hexuloses, yellow; 6-deoxyaldohexoses, yellow or yellow-pink. The background of the plate remained unchanged.

Sprayed phosphate plates were heated at 110° for 20 min. Hexoses showed yellow and pentoses pink. The background of this plate also remained unchanged.

Quantitative Spray:

Pridham's (1956) p-anisidine reagent, devised for paper work gave on both acetate and phosphate plates a stable colour which could be extracted from the Kieselguhr. It consists in 4-methoxy-aniline (p-anisidine) hydrochloride (1 g) dissolved in MeOH (5 ml) containing sodium dithionite (100 mg) and freshly distilled butan-1-ol (95 ml). After spraying, the acetate plates were heated at 130° for 15 minutes when all classes of sugar showed as brownish spots. Phosphate plates were heated at 125° for 15 minutes when hexoses show up yellow grey and pentoses sepia colours thus differing slightly from the colours on acetate plate. There was no background colour on both plates after heating but after 2 - 3 h the acetate plate begins to show grey colour.

The above reagent gives selective colours when used on paper (Pridham, 1956).

$R_F$ Values of authentic sugars

After chromatographic development the position of different sugar spots can be expressed by $R_F$ Value which is the fundamental measurement in chromatography and is defined as follows:-

$$R_F = \frac{\text{distance of centre of substance from the origin}}{\text{distance of solvent travel from the origin}}$$

Ten monosaccharides spotted at 1 cm distances on the acetate and phosphate plates gave the following results:-

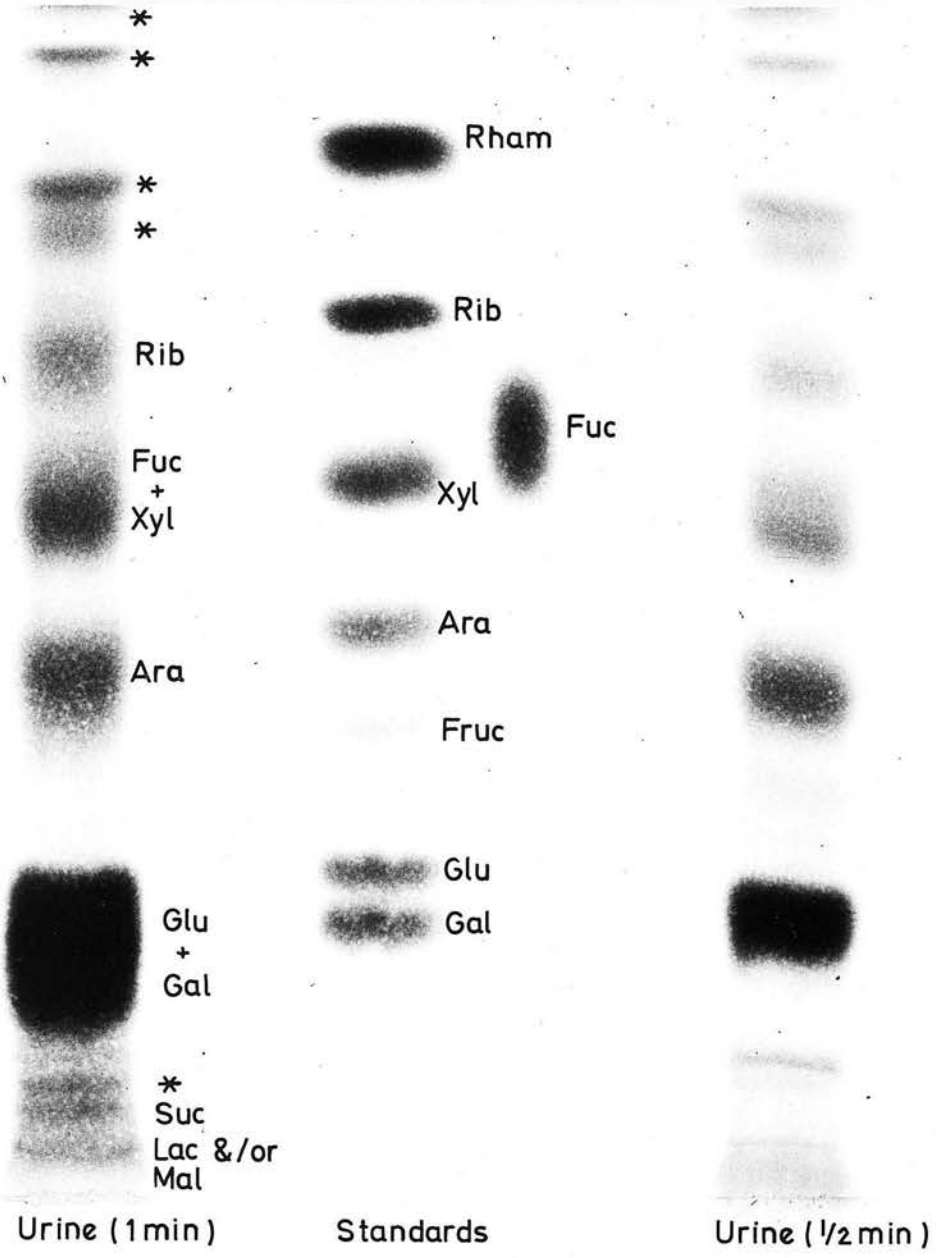
Acetate plates:

With a single development, arabinose did not separate from fructose and/or mannose. Fructose and mannose ran at the same rates and exhibited the same colours with both sprays. With a double development (fig. 9) galactose, glucose, fructose, arabinose, xylose, ribose and rhamnose were completely separated from each other but fucose covered upper $\frac{1}{2}$ of the xylose spot and mannose coincided <u>in toto</u> with the fructose spot.

Four 20 x 20 cm acetate plates were run simultaneously spotting on each plate a mixture containing galactose, glucose, fructose, mannose, arabinose, xylose, fucose, ribose and rhamnose and the $R_F$ values were measured (Table 5).

Phosphate plates:

Separation of fucose from xylose and all other sugars is effected on this system with a single development. But double development markedly increases the $R_F$ values with either solvents 1 or 2 (page 53 ), and the sugar spots became more compact and well shaped. In this system galactose, glucose, fructose, arabinose,




(Fig. 9. See opposite page.)

Fig. 9. T.l.c. on acetate plate (20 x 20 cm) after double development. The middle strip shows the separation of authentic sugars ( 15 µg each); fucose coincided with xylose.

Two lateral strips show extent of separation of sugars in urine volumes of 1 min and ½ min excretion from a normal fasting individual.

* Unknown sugars.

The chromatogram was sprayed with PABA reagent.

xylose and rhamnose were completely separated from each other but ribose coincided with xylose and mannose again coincided with fructose, in toto (fig. 10; Table 6).

TABLE 5

$R_F$ values of 9 monosaccharides from four (20 x 20 cm) acetate plates (run simultaneously) after double development in ethyl acetate-propan-2-ol-water (4:1:0.5)

| | $R_F$ values x 100 | | | |
|---|---|---|---|---|
| Sugars | Plate 1 | Plate 2 | Plate 3 | Plate 4 |
| Galactose | 11 | 12 | 12 | 14 |
| Glucose | 14 | 16 | 15 | 19 |
| Fructose and Mannose | 25 | 26 | 30 | 28 |
| Arabinose | 32 | 34 | 39 | 35 |
| Xylose * | 45 | 46 | 52 | 47 |
| Fucose * | 47 | 48 | 53 | 49 |
| Ribose | 66 | 61 | 67 | 62 |
| Rhamnose | 78 | 76 | 83 | 80 |

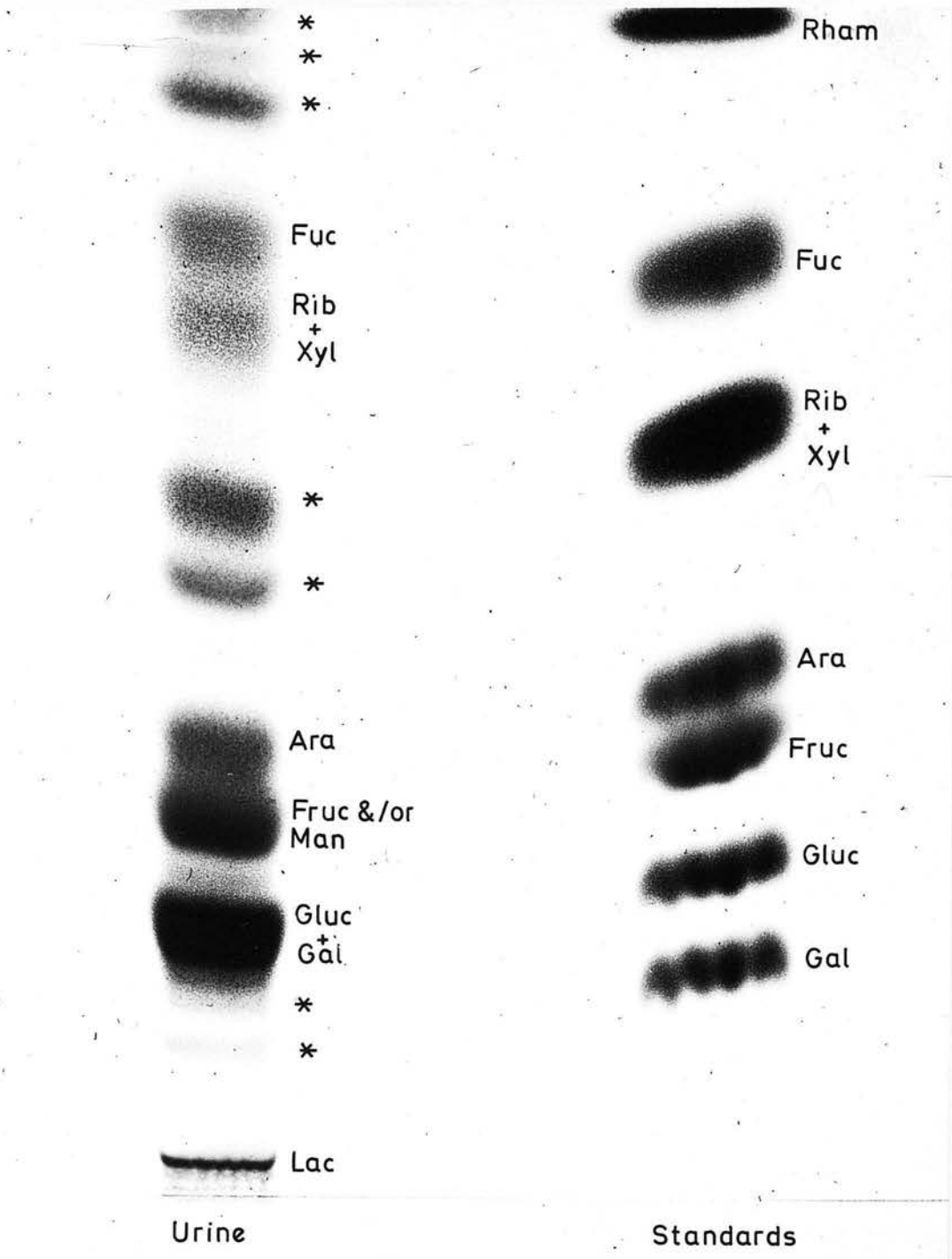
*Fucose and xylose do not separate.

Fig. 10. T.l.c. on phosphate plate (20 x 20 cm) after double development: one strip shows separation of fucose from other authentic (standard) sugars ( 15 µg each); xylose coincided with ribose.

The other strip shows extent of separation of sugars in urine volumes of 1 min excretion from a normal fasting individual.

* Unknown sugars.

The chromatogram was sprayed with Pridham's reagent.




(Fig. 10. See opposite page.)

TABLE 6

Typical $R_F$ values for 9 monosaccharides using a 20 x 20 cm phosphate plate and solvent ethyl acetate-methanol-butan-1-ol-water (16:3:3:2)

| Sugars | $R_F$ x 100 | |
|---|---|---|
| | Single development | Double development |
| Galactose | 10 | 18 |
| Glucose | 14 | 26 |
| Fructose and Mannose | 20 | 34 |
| Arabinose | 25 | 42 |
| Xylose * | 38 | 60 |
| Ribose * | 39 | 62 |
| Fucose | 55 | 77 |
| Rhamnose | 80 | 97 |

* xylose and ribose do not separate.

The above system allows quantitative measurements of fucose as well as of galactose, glucose, fructose and arabinose. Rhamnose runs with the solvent front ($R_F$ 0.97) and is hence unsuitable for quantitative measurement of this sugar.

On a plate 35 cm long and with two developments with solvent 3 (page 53) the eight standard monosaccharides were clearly separated as shown by $R_F$ values (Table 7) and fig. 11.

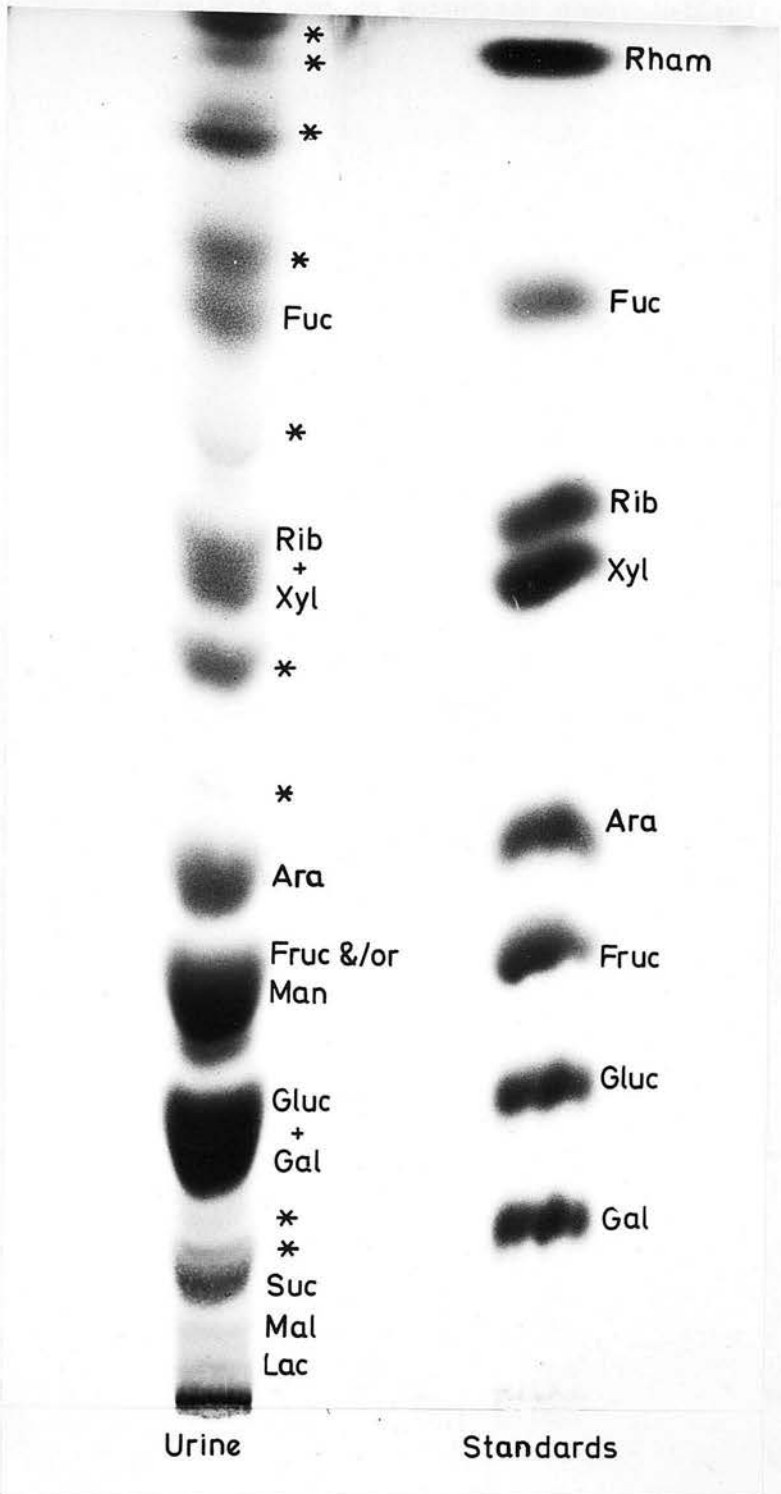
With this procedure however not more than 15 µg each of

Fig. 11. T.l.c. on phosphate plate (20 x 35 cm) after double development showing extent of separation of authentic (standards) sugars ( 15 µg each) and of the sugars in urine volume of 1 min excretion from a normal fasting individual.

Authentic xylose separated from authentic ribose, while, in urine, these sugars failed to separate.

* Unknown sugars.

The chromatogram was sprayed with Pridham's reagent.




(Fig. 11. See opposite page.)

standard xylose and ribose can be separated quantitatively. The longer plate has other disadvantages because each run takes $3\frac{3}{4}$ h and with intermediate drying the whole operation occupies 9 h. Moreover the plate is cumbersome for routine clinical laboratory work.

TABLE 7

Typical $R_F$ values for eight monosaccharides on a 20 x 35 cm plate: Developed twice with solvent 3.

| sugars | $R_F$ x 100 |
|---|---|
| Galactose | 28 |
| Glucose | 42 |
| Fructose and Mannose | 53 |
| Arabinose | 61 |
| Xylose | 77 |
| Ribose | 83 |
| Fucose | 91 |
| Rhamnose | 98 |

Detection of urinary sugars

Criteria of detection or specificity criteria:

That the urinary sugars detected and subsequently measured were arabinose, xylose, ribose and fucose were determined by the following criteria:

i) Sugar spots were individually separated without any

interference by other sugar or coloured spot(s).

ii) Travel distances ($^{R}F$ values) of the urinary sugars were compared with the authentic sugars.

iii) Colours of the urinary sugars were compared with the authentic sugars.

iv) Authentic sugars were run with the urine sample in the same spot and the corresponding sugars were found to travel in the same place.

Acetate plates:

Photographic representations (figs. 9-11) are not discriminating for all the sugars actually present on the chromatogram. However, when smaller amount of urinary concentrate (equivalent to $\frac{1}{2}$ min excretion of urine) was chromatographed, the spots were more discrete and a diagrammatic representation of a typical fasting urine sample is shown in fig. 12. With the criteria described above, galactose, glucose, fructose and/or mannose, arabinose, xylose, fucose and ribose were detected. The sugar spot which might be suspected to be rhamnose (unknown spot just above ribose spot in fig. 12) was probably not in fact that sugar. This constant urinary sugar presented a slightly different colour from rhamnose which was only noticed on close observation. An attempt to identify this sugar has so far met with no success. It proved to be none of the following 6-deoxyhexoses: 6-deoxy-D-allose; 6-deoxy-D-glucose; 6-deoxy-D-talose; 6-deoxy-L-idose; 6-deoxy-D-gulose (Specimens of these sugars were kindly supplied by

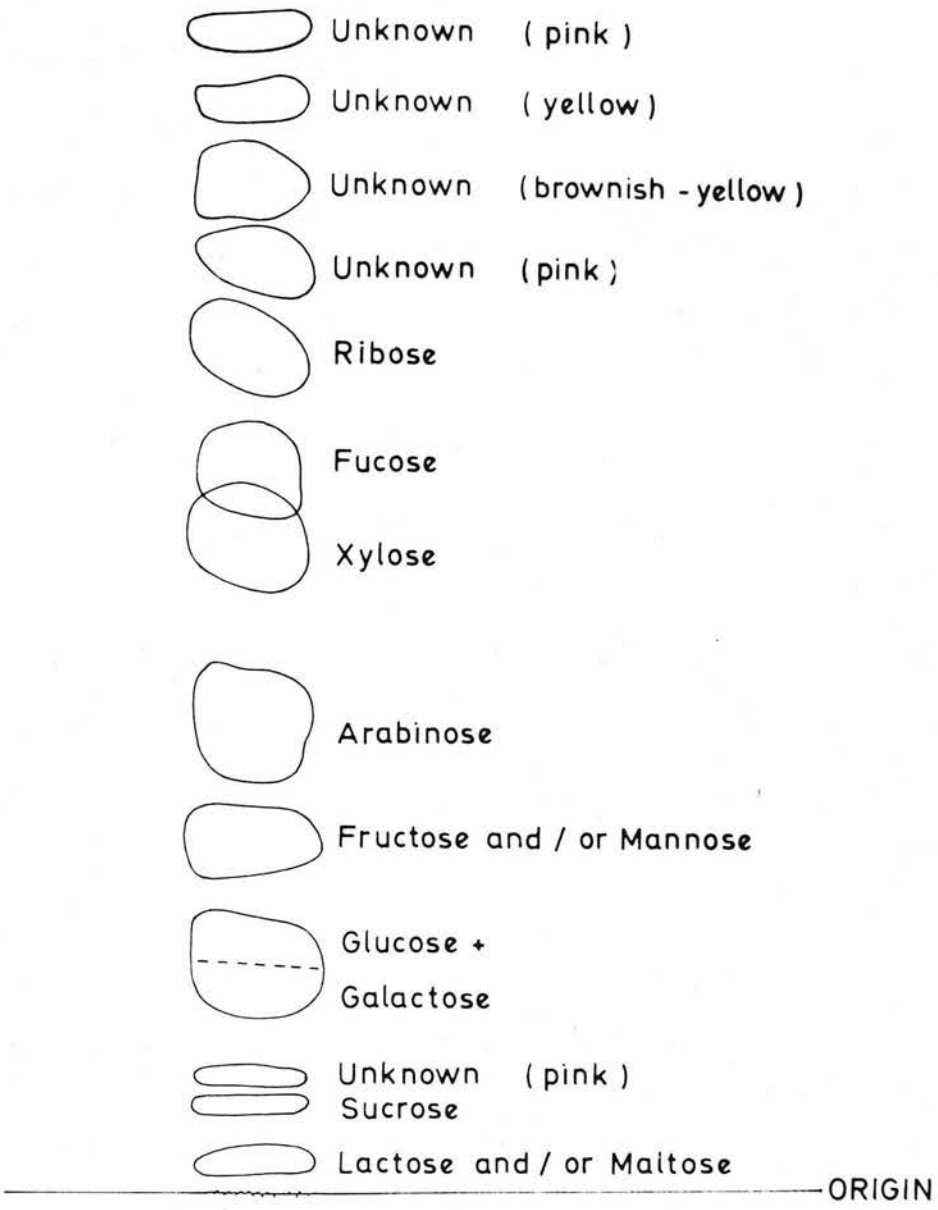



Fig. 12 Diagrammatic representation of the fasting urinary sugar spots on thin-layer acetate plate (20 x 20 cm) after double development when sprayed with PABA or Pridham's reagent.

Prof. T. Reichstein of Basel); nor it was sedoheptulosan (sedoheptulose will be largely dehydrated to its anhydride under my experimental conditions).

Although galactose and glucose standards (upto 80 µg each) show good separation, but when urine is examined, this separation is not complete (fig. 9). Urinary arabinose and ribose are spatially well separated and these two sugars were measured on this plate (fig. 9).

Spot 1 (first spot above the origin in fig. 12) could be lactose and/or maltose. When run separately these two sugars on this system showed the same distance of travel and same colour (Yellow). Spot 2 (fig. 12) was detected as sucrose.

A slightly slower movement of the urinary sugars in comparison with the authentics could be due to the presence of urinary polyols (cf. Shellard & Jolliffe, 1966) which have been found in urine, in appreciable amounts (Touster et al., 1960; Pitkanen et al., 1964).

Fast running pink sugar(s) (No. 13 in fig. 12) could be glucuronolactone and/or pentuloses. The unknown sugars are not lyxose, apiose or allulose.

Phosphate plates:

On this plate also, a smaller amount of urinary concentrate ($\frac{1}{2}$ min excreted urine) was chromatographed for better discrimination of the sugar spots. Diagrammatic representation of a typical fasting urine sample is given in fig. 13. Lactose and maltose were found to

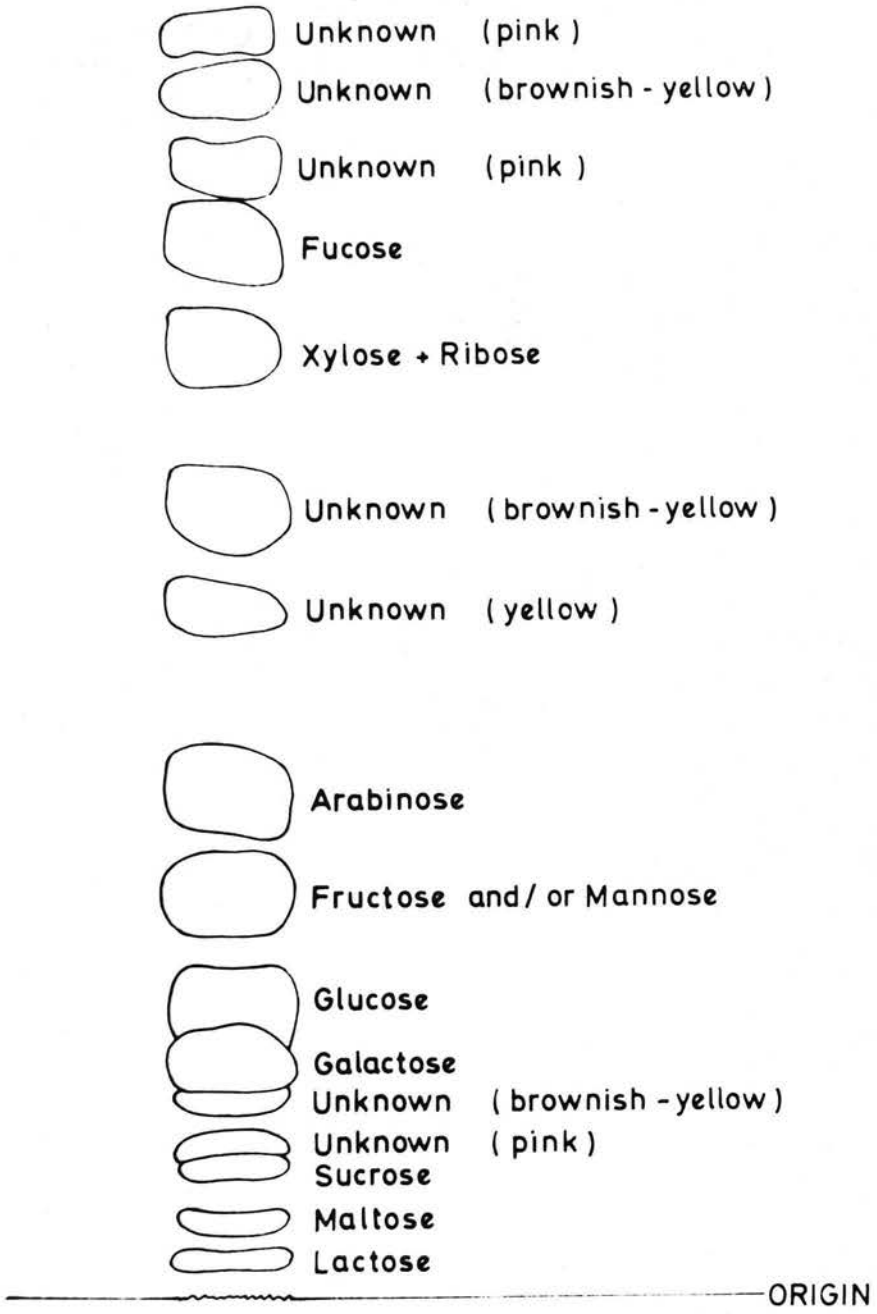



Fig. 13. Diagrammatic representation of the fasting urinary sugar spots on thin-layer phosphate plate (20 x 20 cm) after double development and when sprayed with PABA or Pridham's reagent.

separate but quantitative analysis of these sugars on this system is not possible. The upper margin of fucose is just touched by another unknown sugar (fig. 13) but the two sugars could be sufficiently discriminated and no difficulty is encountered in scraping off the fucose spot.

The unknown sugars are not lyxose, apiose or allulose.

Separation of urinary aldopentoses and fucose in relation to the amount of urine applied on the t.l. plates.

Routinely one minute excreted urine was spotted on the acetate plate and two minutes on phosphate plate. More than this amount of normal urine on acetate plate caused sharing of lower margin of arabinose with upper margin of fructose and/or mannose and upper margin of ribose with lower margin of the unknown sugar (figs. 9 & 12). Similarly on phosphate plate, if more than 2 min excreted urine was applied there was merging of the margin of xylose+ribose with the unknown sugar below and upper margin of fucose with the lower margin of the unknown sugar above (figs. 10 & 12). On both acetate and phosphate plates, upto 80 µg of standard sugars were quantitatively separated; but when applied to urine, fructose and/or mannose tend to share margin with arabinose with much lower concentration of each. This is presumably due to the presence of the unknown sugars 'trying to squeeze in where there is no sufficient room for them'. Moreover the polyols perhaps held back the travel distance and hinders the separation of urinary sugars (page 66 ).

## QUANTITATIVE MEASUREMENTS

### Removal of spots for spectrophotometry

Because the sugar spots were well separated it was possible completely to scrape off, using a razor blade, the coloured areas of the Kieselguhr from the chromatograms. Equal areas of the Kieselguhr which contained the stain were carefully transferred to centrifuge tubes; at the same time equal areas which contained no sugars were treated likewise to serve as blanks (page 79 ).

### Extraction of the colours

For spectrophotometric measurements the colours developed by Pridham's reagent and PABA reagent on both acetate and phosphate plates were extracted within half an hour of colour development as follows:-

### A. Stained by Pridham's reagent

Acetate plate:

To each tube containing the areas of Kieselguhr-G with or without sugar spots, was added 4 ml of 95% aqueous methanol containing 1 g of $SnCl_2$/100 ml. The tubes were stoppered and vigorously shaken for 5 min and the solid then packed on the centrifuge. The supernatant solutions were then directly transferred into the 'cells' to be measured at 395 nm ($\lambda$ max), against the "blank" supernatant on an SP500 spectrophotometer. The absorption spectrum between 350 and 650 nm is shown in fig. 14. The colour was stable for 3 h and the three aldopentoses gave almost identical extinctions

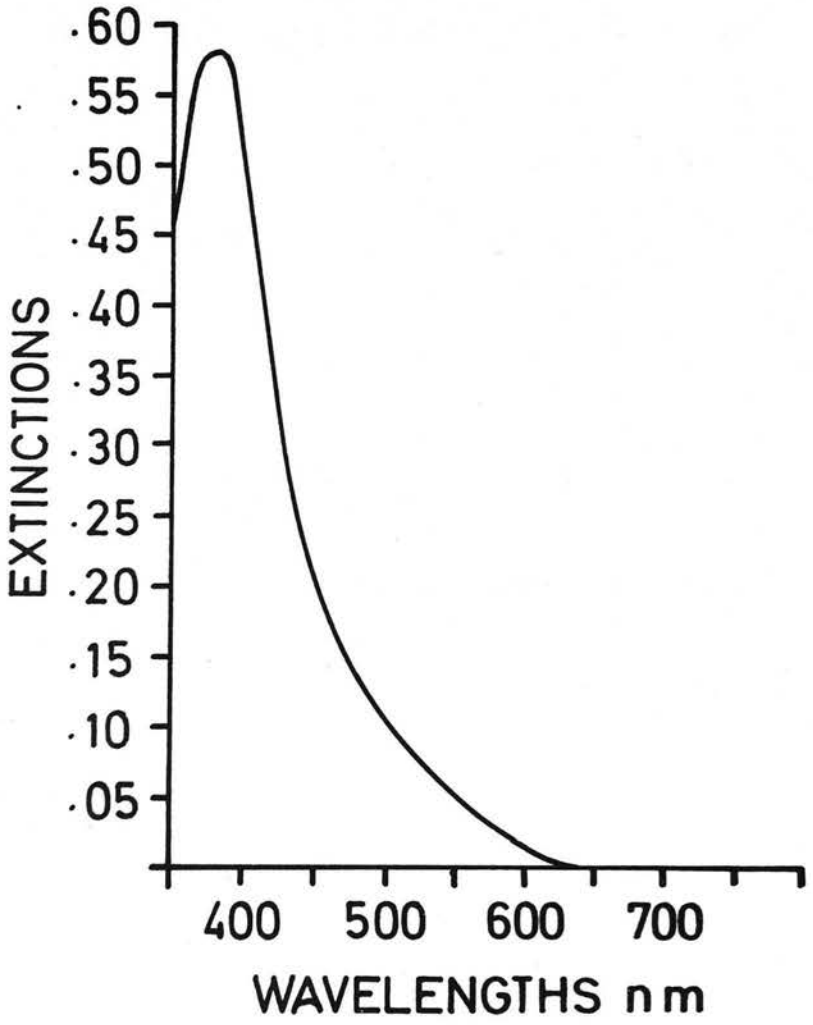



Fig. 14. Typical peak absorption of aldopentoses (40 µg/4ml) recovered from acetate plate after spraying with Pridham's reagent.

in the range of 5 to 80 µg. Typical results are given in Table 8 and linearity in fig. 15.

TABLE 8

Typical extinctions for three aldopentoses from acetate plate stained by Pridham's reagent.

| Amount (µg) | Extinctions | | |
|---|---|---|---|
| | Arabinose | Xylose | Ribose |
| 5 | 0.045 | 0.045 | 0.040 |
| 10 | 0.12 | 0.12 | 0.09 |
| 20 | 0.24 | 0.25 | 0.23 |
| 40 | 0.58 | 0.58 | 0.60 |
| 80 | 1.15 | 1.15 | 1.16 |

Phosphate plate:

The procedure was identical with that used above. Equal areas of the adsorbent containing the coloured spots were scraped off; the colour was extracted by shaking with 3 ml of a mixture of methanol (90 ml) and 5 ml of 1% (w/v) aqueous stannous chloride. From this plate however, extinctions were measured at 380 nm ( $\lambda$max) in an SP500 spectrophotometer. Fig. 16 shows the peak absorption spectrum of xylose, ribose and fucose between 350 and 900 nm. The colour was stable indefinitely. Table 9 shows typical extinctions and fig. 17 shows the linearity in the range of 5 µg to 80 µg for fucose

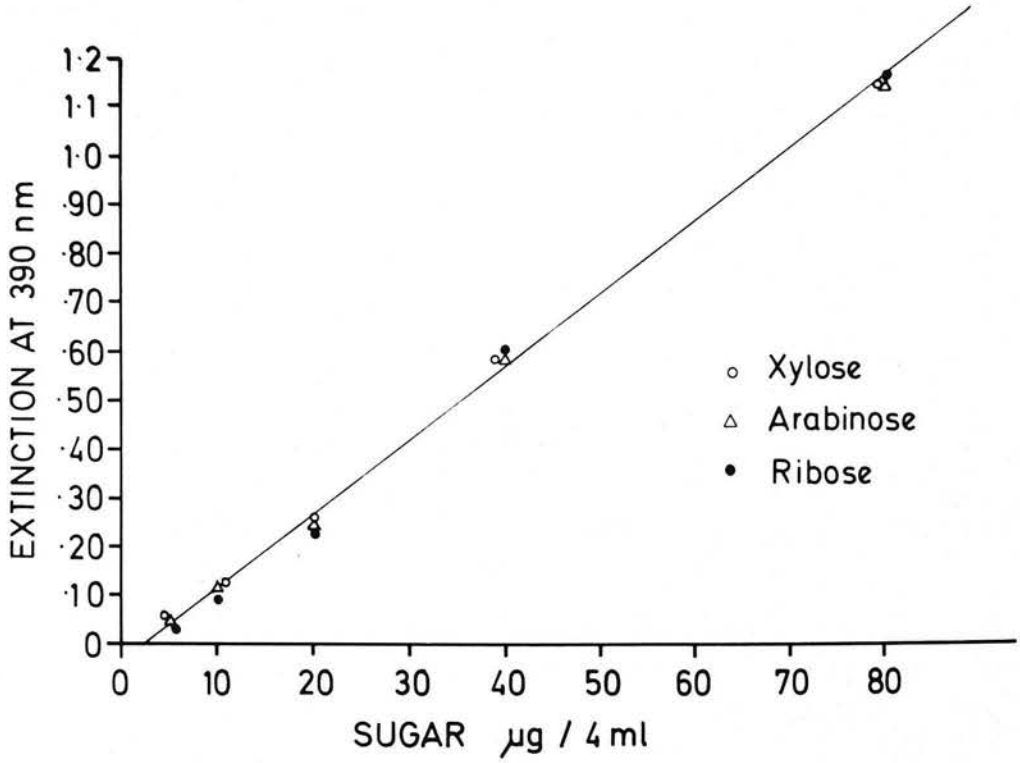



Fig. 15. Aldopentoses. Pridham's spray. Regression line showing linearity of recovered colour development on acetate plates. Note (a) This line does not pass exactly through the origin (b) the method cannot be used below a concentration of 2.5 µg/4 ml.

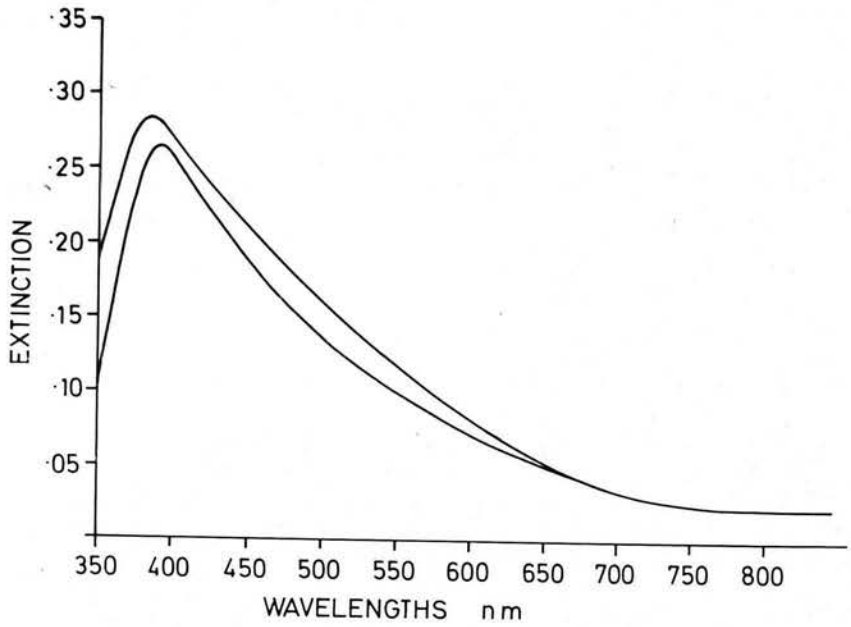



Fig. 16. Peak absorption of xylose & ribose (40 µg/3 ml) (upper curve) and fucose (40 µg/3 ml) (lower curve) recovered from phosphate plates after spraying with Pridham's reagent.

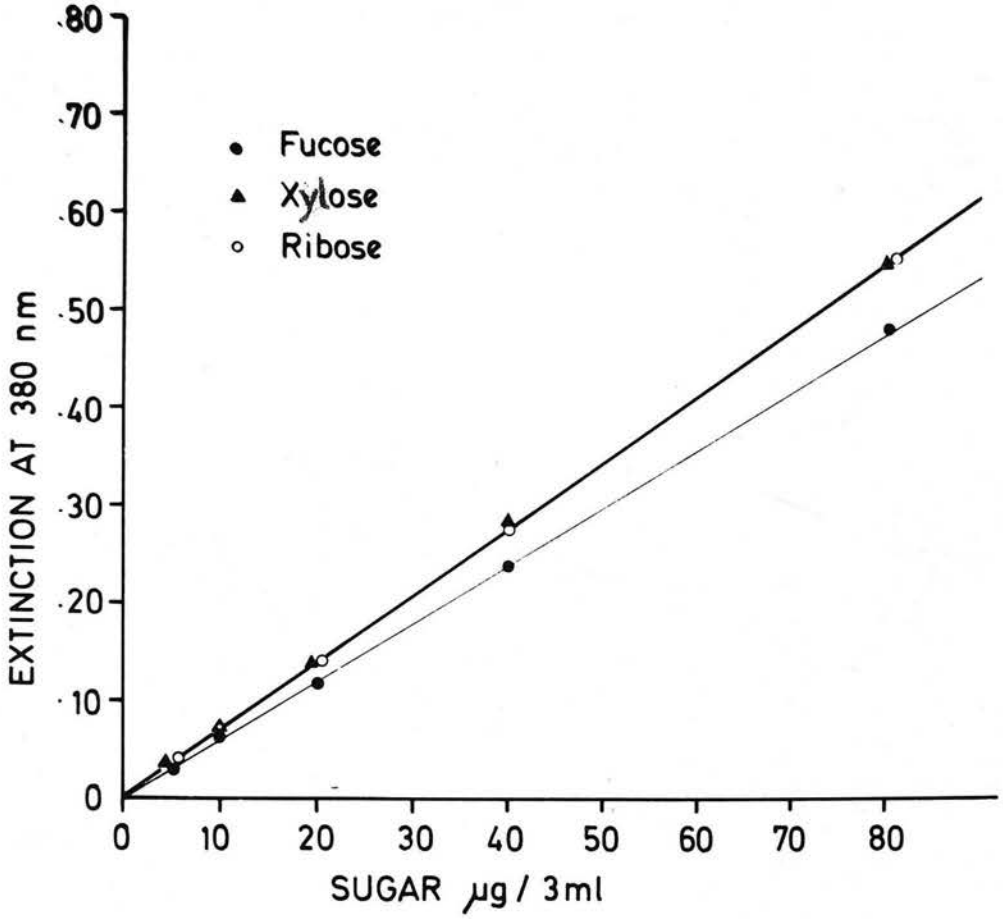



Fig. 17. Typical calibration curves showing linearity of fucose, xylose and ribose extracted from colour development on phosphate plate using Pridham's reagent.

and also for xylose and ribose run separately under the same conditions.

TABLE 9

Typical extinctions at 380 nm for fucose and two aldopentoses stained by Pridham's reagent.

| Amount (µg) | Extinction | | |
|---|---|---|---|
| | Fucose | Xylose | Ribose |
| 5 | 0.03 | 0.035 | 0.035 |
| 10 | 0.065 | 0.075 | 0.07 |
| 20 | 0.125 | 0.14 | 0.14 |
| 40 | 0.24 | 0.275 | 0.28 |
| 80 | 0.48 | 0.55 | 0.55 |

Determination of xylose

The xylose spot coincided with fucose on the acetate plate and ribose on the phosphate plate. So the xylose value was determined in the following way. Amount of ribose was determined from the acetate plate which was then subtracted from the (xylose + ribose) value obtained from phosphate plate since both pentoses yielded the same amount of colour, weight for weight (Table 9).

B. Stained by PABA reagent

Acetate plate:

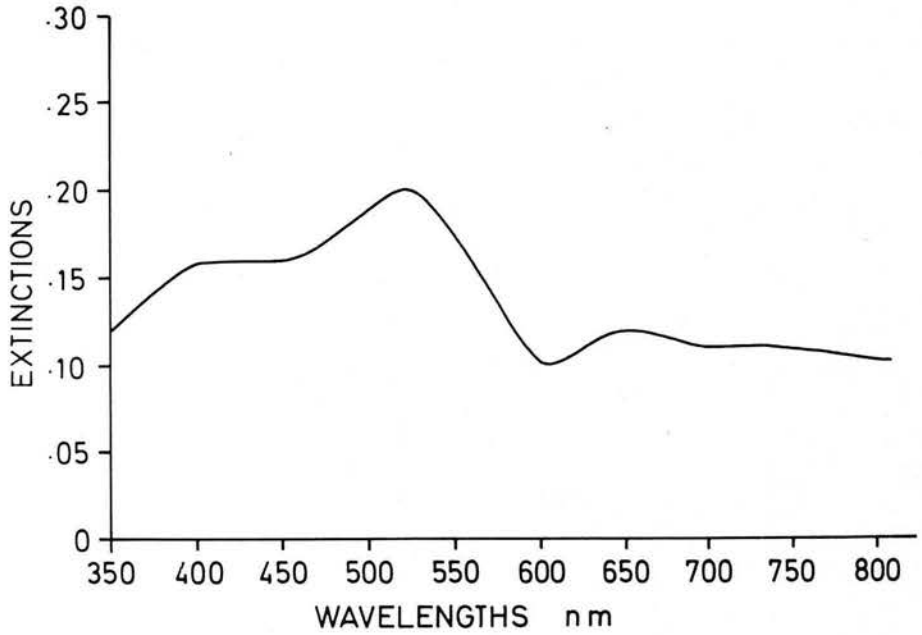



Fig. 18. Peak absorption of xylose (20 µg/3 ml) recovered from acetate plate after staining with PABA reagent.

The extractant solvent used was 3 ml of 80% aqueous acetic acid containing 2 g of $SnCl_2$ per 100 ml. Scraped Kieselguhr G with or without sugar spots in the centrifuge tubes were shaken vigorously with the above solvent for 5 min. The clear cerise supernatant obtained after centrifugation was read against blank at 520 nm ( $\lambda$max) (absorption spectrum in fig. 18). The colour was only stable for two hours. Typical extinctions of xylose in the range 5 to 80 µg are given in Table 10 and linearity in fig. 19. Extinctions for arabinose and ribose were not measured.

TABLE 10

Typical extinction at 520 nm for xylose from acetate plate stained by PABA reagent.

| xylose µg | Extinctions |
|---|---|
| 5 | 0.06 |
| 10 | 0.105 |
| 20 | 0.215 |
| 30 | 0.29 |
| 40 | 0.33 |
| 80 | 0.36 |

Phosphate plate:

The extractant solvent and the procedure were same as in acetate plate above. The yellowish brown supernatant solution was read against a blank at 380 nm ( $\lambda$ max) (absorption spectrum in fig.

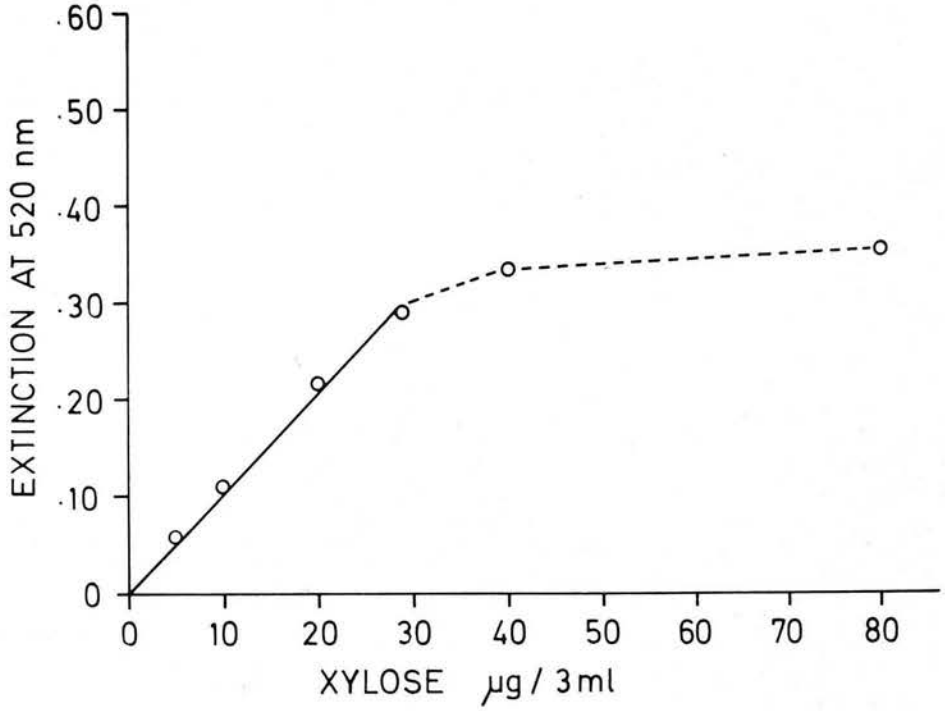



Fig. 19. Typical calibration curve showing linearity of xylose recovered from acetate plate after staining with PABA reagent.

Note: Linearity is lost and the curve became flat beyond the concentration of 30 µg/3 ml (shown by the interrupted lines).

20). The colour was stable for at least 6 h. Typical extinction of fucose in the range of 5 to 80 µg are given in Table 11 and linearity in fig. 21. Extinctions for aldopentoses were not measured.

TABLE 11

Typical extinction at 380 nm for fucose from phosphate plate stained by PABA reagent

| fucose µg | Extinction |
|---|---|
| 5 | 0.04 |
| 10 | 0.085 |
| 20 | 0.165 |
| 40 | 0.30 |
| 80 | 0.60 |

Blank values

Blank values of sugar free areas of Kieselguhr G were obtained by measuring against the appropriate extractant solvent and were repeatedly found to range between E 0.005 and 0.01 from acetate plate and E 0.01 and 0.02 from phosphate plate.

Choice of quantitative spray reagent

Pridham's spray reagent was preferentially used for the quantitative measurement on the thin-layer of the urinary aldopentoses and fucose.

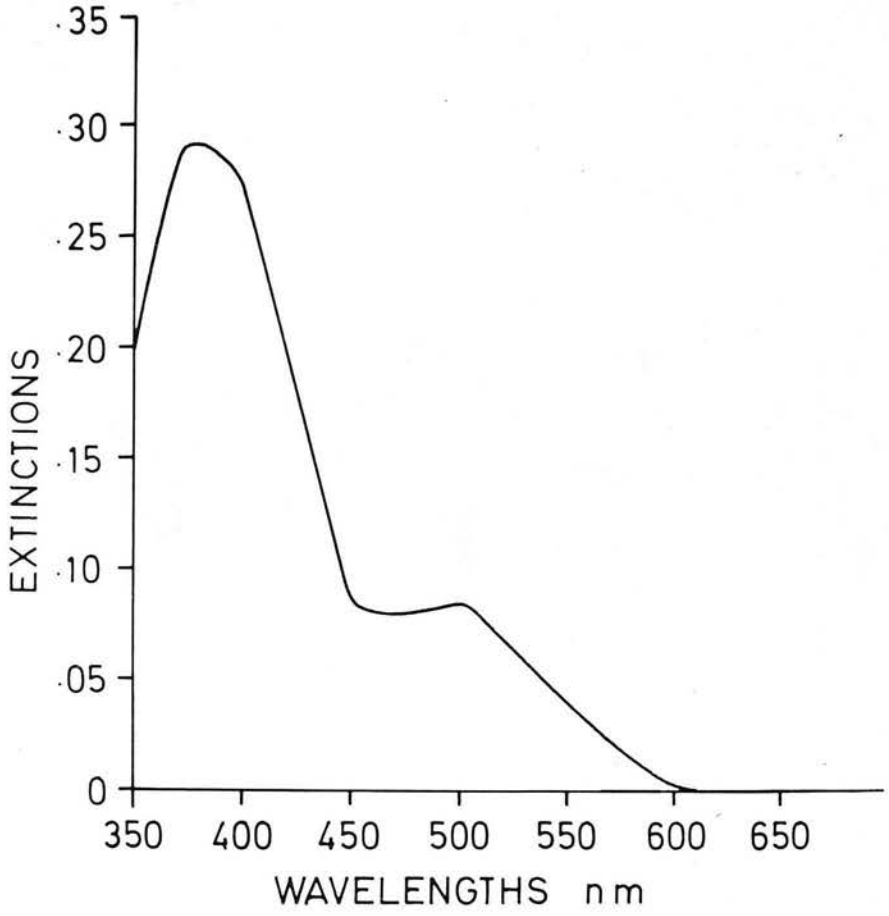



Fig. 20. Peak absorption of fucose (40 µg/3 ml) recovered from phosphate plates after staining with PABA reagent.

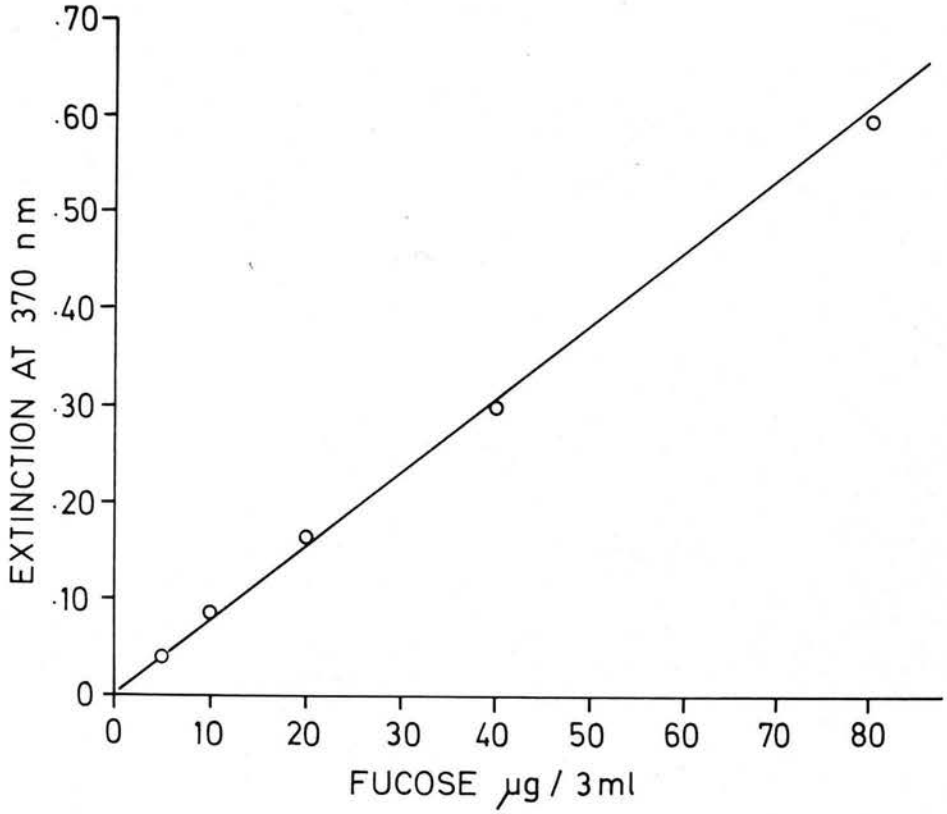



Fig. 21. Typical calibration curve showing linearity of fucose extracted from phosphate plate staining with PABA reagent.

Amount of standards used and variation in extinction

On acetate plate the extinctions of the three aldopentoses at different concentrations were practically same (Table 8, fig. 15). Arabinose and ribose were measured in urine samples on acetate plate against the average extinction of each of 15 µg of the sugars. Similarly on phosphate plate E of ribose and xylose at different concentration was same but of fucose was slightly lower. Xylose+ ribose in urine sample were measured against the average extinction of two spots of 30 µg of xylose and fucose against the average extinction of two spots of 30 µg of the sugar.

The extinction of 15 µg of standard pentoses on acetate plate usually ranged between E 0.12 and 0.20 and on phosphate plate for each 30 µg of fucose and xylose ranged between E 0.15 and 0.24.

Reliability of the methods

I. Linearity:

With Pridham's spray reagent and after extracting colours with methanol and $SnCl_2$, a linear relationship was found upto 80 µg of each sugar (figs. 15 and 16). On regression analysis showing linearity for the three aldopentoses on acetate plate it was noted that the line did not pass through the origin. On acetate plate a linear relationship was found upto 30 µg of xylose with PABA spraying reagent and extracting with acetic acid and $SnCl_2$. On phosphate plate the E of fucose hold linear relationship upto 80 µg. The only other work which could be compared was on thin-layer of

cellulose by Wolfrom et al. (1966) who obtained linearity upto 150 ug of certain monosaccharides (there is no mention of E).

II. Reproducibility:

(i) Qualitative: On both acetate and phosphate plates, though $R_F$ value varied from plate to plate (as in Table 5) under the conditions described, the separation of the sugars was repeatedly reproducible.

(ii) Quantitative: The thin-layer chromatographic procedure on acetate and phosphate plate was applied for the measurement of urinary sugars of human subject. As a preliminary investigation the same sample of a fasting urine was subjected to seven separate measurements using separate column for each for deionisation.

The results on seven occasions are given in Table 12.

TABLE 12

Reproducibility in a septuplicate analyses of a single urine sample

$$\text{Co-efficient of variation} = \frac{SD}{Mean} \times 100$$

| Analytical treatment | Excretion µg/min | | | |
|---|---|---|---|---|
| | Ara | Xyl | Rib | Fuc |
| 1 | 16 | 10 | 8 | 28 |
| 2 | 21 | 9 | 9 | 27 |
| 3 | 23 | 14 | 8 | 27 |

TABLE 12 (Cont.)

| Analytical treatment | Excretion µg/min | | | |
|---|---|---|---|---|
| | Ara | Xyl | Rib | Fuc |
| 4 | 24 | 11 | 7 | 34 |
| 5 | 22 | 11 | 8 | 32 |
| 6 | 24 | 12 | 8 | 34 |
| 7 | 21 | 13 | 8 | 31 |
| Coeff. of variations | 13.1% | 15% | 7.2% | 10.2% |

III. Recovery of sugars from urine:

Known amount of pure arabinose, xylose, ribose and fucose at different concentrations (ranging from 10 µg to 30 µg of each sugar/ml of 5 ml urine used each time in a column) were added to the same urine sample and passed through six different columns for deionisation. Another six columns were set as control through which only the urine sample was run. An arbitrary pair of columns was set (one with pure sugar and urine and another with urine only). The result obtained from the urine sample was subtracted from the sample containing pure sugar and urine. The recovery of the four sugars obtained on six different occasions are given in Table 13 to 16.

TABLE 13

Recovery of arabinose from urine

| Pure arabinose added µg | Urinary arabinose+ pure arabinose estimate µg | Urinary arabinose µg | Recovery estimate of added arabinose µg | % |
|---|---|---|---|---|
| 10 | 28 | 20 | 8 | 80 |
| 10 | 31 | 23 | 8 | 80 |
| 20 | 45 | 24 | 21 | 105 |
| 20 | 38 | 22 | 16 | 80 |
| 30 | 58 | 24 | 34 | 113 |
| 30 | 52 | 21 | 31 | 103 |

TABLE 14

Recovery of xylose from urine

| Pure xylose added µg | Urinary xylose+ pure xylose estimate µg | Urinary xylose µg | Recovery estimate of added xylose µg | % |
|---|---|---|---|---|
| 10 | 14 | 9 | 5 | 50 |
| 10 | 26 | 14 | 12 | 120 |
| 20 | 27 | 11 | 16 | 80 |
| 20 | 26 | 11 | 15 | 75 |
| 30 | 46 | 12 | 34 | 113 |
| 30 | 63 | 13 | 50 | 166 |

TABLE 15

Recovery of ribose from urine

| Pure ribose added µg | Urinary ribose+ Pure ribose estimate µg | Urinary ribose µg | Recovery estimate of added ribose µg | % |
|---|---|---|---|---|
| 10 | 20 | 9 | 11 | 110 |
| 10 | 17 | 8 | 9 | 90 |
| 20 | 25 | 7 | 18 | 90 |
| 20 | 25 | 8 | 17 | 85 |
| 30 | 37 | 8 | 29 | 96 |
| 30 | 38 | 8 | 30 | 100 |

TABLE 16

Recovery of fucose from urine

| Pure fucose added µg | Urinary fucose+ Pure fucose estimate µg | Urinary fucose µg | Recovery estimate of added fucose µg | % |
|---|---|---|---|---|
| 10 | 35 | 27 | 8 | 80 |
| 10 | 35 | 27 | 8 | 80 |
| 20 | 56 | 34 | 22 | 110 |
| 20 | 54 | 32 | 22 | 110 |
| 30 | 64 | 34 | 30 | 100 |
| 30 | 58 | 31 | 27 | 90 |

The mean±S.D. recoveries of arabinose, xylose, ribose and fucose from six estimations were 93%±15%, 101%±41%, 95%±9% and 95%±14% respectively. The range of recoveries of the above sugars were respectively 80-113%, 50-166%, 85-110% and 80-110%. The wide range in the recovery of xylose appears to be due to the indirect method involved in its estimation. The co-efficient of variation was also highest for xylose. Date (1958a, 1958b) obtained recovery of xylose and arabinose between 92 to 102% after passing through ion exchange resin columns and subsequent paper chromatography.

IV. Sensitivity:

Qualitatively 0.5 μg of any sugar in the present study was detectable on either t.l. chromatogram with the spraying reagents described.

Quantitatively, although on the acetate plate the linear relationship held between E 0.04 (5 μg) and 1.16 (80 μg) for the aldopentoses, the lowest limit of spectrophotometric estimation for any of the sugars was 2.5 μg. This is taken arbitrarily from regression analysis showing linearity of recovered colour development on acetate plate (fig. 15). On the phosphate plate, however, the sensitivity is somewhat low where linear relationship held between E 0.03 (5 μg) and 0.55 (80 μg) for fucose, xylose and ribose, although the linear curve pass through the origin.

Because of low urinary excretion value of ribose (pages 125, 126) this sugar was measured at the lower limit of the linear curve.

This could be improved by applying 2 min excreted urine on the acetate plate but would in turn hinder the separation of arabinose from fructose and/or mannose.

Ideally, spectrophotometric measurements should be carried out around the mid-way extinction value of a linear curve. However this was not possible in the present study because of obvious limitations as mentioned above.

# CHAPTER 4

I. SELECTIVE REVIEW OF THE EXISTING METHODS OF SEPARATION AND ESTIMATION OF ALDOPENTOSES AND FUCOSE.

II. DISCUSSION: THE NEW QUANTITATIVE METHODS.

# SELECTIVE REVIEW OF THE EXISTING METHODS OF SEPARATION AND ESTIMATION OF ALDOPENTOSES AND FUCOSE

## Introduction:

Analyses, both qualitative and quantitative of mixtures of monosaccharides of biological interest have been approached in many ways. The following precis of methods commonly applied gives a general picture of procedures adopted before and after the present t.l.c. system was devised. It should be pointed out here that few if indeed any of the published methods will satisfactorily separate all one from another and from other sugars, those monosaccharides with which this dissertation is concerned.

## A. Column chromatography

Column chromatography separates by selective adsorption, a mixture of substances into zones or bands on a column of solid powdered materials after the passage of appropriate solvents. A partition column employs partition of substances between two immiscible liquid phases and may be considered as a one-dimensional chromatogram where the supporting solid phase is contained within a tube and is the real ancestor of both paper and t.l.c.

### 1. Carbon:

Selective elution of classes of sugars (mono-, di- and trisaccharides) from charcoal columns was first described by Whistler

& Durso (1950). Hughes & Whelan (1958) showed that a special variety of charcoal could be used to separate mono- and higher saccharides in relation to their molecular weights. Such systems were reasonably quantitative down to the milligram level.

2. Cellulose:

Hough et al. (1949) using a cellulose column eluted with butan-1-ol saturated with water, separated a mixture containing 50 mg each of L-rhamnose, D-ribose, L-arabinose and D-galactose.

3. Purified potato starch:

On eluting columns of purified potato starch with butanol-propanol-water, Gardell (1953) separated seven monosaccharides, galactose, glucose, mannose, xylose, ribose, fucose and rhamnose each in amounts of 100 to 200 μg.

4. Ion-exchange chromatography of carbohydrate-borate complex and "high resolution" column chromatography:

Khym & Zill (1952) separated several mono- and disaccharides on a column of a strong-basic resin in its borate form on which sugars acquire a negative charge. A marked and automated improvement of this technique by Kesler (1967) gave separation in 4 to 6 h and quantitative measurement of mono- di- and trisaccharides at the microgram level. Arabinose, xylose and ribose, but not fucose, were included in a mixture of 17-components. A similar quantitative analysis of saccharides, including fucose, was made by

Walborg & Lantz (1968) using Dowex resin and boric acid-glycerol buffers at a lower pH (6.8) to avoid any of the alkaline re-arrangement reactions reported by Carubelli (1966).

Jolley & Freeman (1968) described an automated carbohydrate analyser consisting of a heated high-pressure anion exchange column to chromatograph borated sugar mixtures. The eluted carbohydrates were detected by sulphuric acid-phenol colorimetry. Jolly et al. (1970) and Young (1970) both used this method to detect and measure sugars in plasma and urine, in non-fasting human subjects.

In the above procedures the chromatographic peaks were identified by the only criteria of comparing their elution times with those of well defined peaks given by known sugars. Applied to biological fluids, such procedures, sometimes involving considerable heating, make it difficult to identify or measure small amounts of sugars with certainty since interference by unsuspected isomers cannot be ruled out.

B. Paper electrophoresis

Electrophoresis of neutral carbohydrates stems from the observation that polyhydroxy compounds react with borate to give anionic complexes which can migrate within a suitable solid matrix when exposed to an electromotive force. Adequate qualitative separations are usually rapid but, because of lateral and longitudinal diffusion migration "dilution" of the spot takes place. This can be minimised if "high voltage" apparatus is available.

Consden & Stanier (1952) first applied paper electrophoresis to fructose, sorbose, glucose, galactose, mannose, ribose, arabinose, rhamnose, cellobiose and raffinose. At 5 different pH values ranging from 7 to 9.7 there was no satisfactory separation of ribose and arabinose from each other and from all other sugars. Since then considerable work has been done (Gross 1953; Hasimoto et al., 1952; Bourne et al., 1956) but satisfactory separation of all the sugars of the present interest was not possible. In electrophoresis applied to urine Marby et al. (1963) detected neither arabinose, xylose nor fucose.

Electrophoresis of sugars in biological fluids was of limited value (Effron 1968), because no single buffer gave good separation of even the limited number of sugars likely to be encountered. Moreover the sensitivity of this method was relatively poor.

C. Partition chromatography by ion-exchange resins

Arwadi & Samuelson (1964) separated mixtures of arabinose, xylose, mannose and galactose (2.5 mg each) and glucose (5 mg) using a column of packed crushed Dowex ($SO_4^{2-}$) and celite with elution by 88% EtOH. The eluate fractions after treating with 0.6 N $H_2SO_4$ were analysed in a 'Technicon Autoanalyser.' Larson & Samuelson (1965) also studied an automated separation of 16 monosaccharides including fucose, ribose, arabinose, xylose by partition chromatography on anion exchange resins.

Cation exchange resins in their lithium, sodium or potassium forms have been used in automated separation of monosaccharides (Jonsson & Samuelson, 1967a). The peak elution volumes of 16 monosaccharides including aldopentoses and fucose were determined in 92.4% ethanol at 75° and 100°. The main advantages of the cation exchanger over the anion exchanger, as stated by the authors, are in analysis of solutions containing deoxysugars and ketoses. Aldopentoses and fucose do not separate well from each other.

Using a strongly basic anion exchanger with a more polar resin matrix, Jonsson & Samuelson (1967b) separated ribose, lyxose, arabinose and xylose (0.5 mg each); fucose was not included.

None of the above separations have been applied to urine. Moreover, relatively much larger amounts of each sugar were examined and the minimum level of detection of the sugar by these methods appears to be high.

D. Gel chromatography

Gel chromatography (gel filtration, gel permeation, molecular sieve chromatography) is based on the decreasing permeability of the three-dimensional network of a swollen gel to molecules of increasing size. There have been no attempts to separate mixtures of various monosaccharides including aldopentoses (Churms, 1970); small differences in $K_d$ among the different sugars (Marsden, 1965)indicate that separation might be achieved only on very large columns. Separation of glucose from ribose and maltose from isomaltose on the

polyacrylamide gel has been reported. However gel filtration methods cannot be successfully employed for the separation of microgram quantities of aldopentoses and fucose.

E. Fluorometry

Fluorometric estimations of free sugars have been mainly confined to glucose in plasma and in urine (Rubin, 1970) and are based on the enzymatic conversion of glucose to glucose-1-phosphate, rearrangement to the 6-phosphate, oxidation to 6-phosphogluconic acid with concomitant production of the fluorescent NADP.2H. Keller (1965) Schersten & Tibbling (1967) thus measured glucose in normal urine. A variety of free sugars react with o-phenylene-diamine (OPD) in strongly acid solutions to form stable fluorescent products which were employed to measure, e.g., arabinose, xylose and ribose (Towne & Spikner, 1963) in artificial mixtures. Fucose was not considered.

F. Gas-liquid chromatography (g.l.c.)

This method ideally requires that a suitable volatile derivative be prepared in quantitative yield from each monosaccharide and that a mixture of the derivatives being analysed be resolved completely. At present this is difficult and usually involves control experiments to determine actual yields. Moreover, free monosaccharides can yield possibly 5 derivatives unless they are previously reduced to the alditols. Without this prior elimination of the "reducing group", analyses become very complicated. Trimethylsilyl ethers were successfully used by Sweeley et al. (1963). Quantitative separations was obtained of mixtures containing, besides

other sugars, ribose and fucose (Richey et al., 1964), arabinose, xylose and ribose (Farshtchi & Moss, 1969) and arabinose, xylose and fucose (Reid et al., 1970).

Sawardekar et al. (1965) and Lehnhardt & Winzler (1968) separated alditol acetates derived from arabinose, xylose, ribose and fucose.

Successful g.l.c. analyses of the neutral sugars obtained after hydrolysis of GGG* and glycopeptides has been reported by numerous workers (e.g. Oates & Schrager, 1967; Lehnhardt & Winzler, 1968; Dawson & Clamp, 1970). Wells (1970) reviewed the methods for measuring carbohydrates in tissues, urine and blood, but no actual application to urine had been made. G.l.c. analyses of carbohydrates in biological materials has been extensively reviewed by Clamp et al. (1971); very little information was given on free urinary sugars. A sample of urine was analysed by Wells et al. (1964); only fructose, glucose and myoinositol with four other unknown peaks were found. Bhatti & Clamp (1968) examined one sample of urine almost certainly from a fed subject (personal communication to Dr. D.J. Bell). Fucose, fructose, mannose, galactose, glucose, N-acetyl-galactosamine, N-acetyl-glucosamine, lactose, sucrose, maltose, cellobiose, trehalose, palatinose and gentiobiose were found but no pentoses were identified. This may have been because this procedure detected sugars in the range of 25 - 500 µg/ml.

A sample of deionised urine from a fasting subject of one of

---

* peptidoglycosaminoglycuronoglycans

the present experiments was kindly analysed several times by Mr. Peter Daniel of the Biochemistry Laboratory, Oxford, to whom the writer is greatly indebted; using O-trimethylsilyl ethers of the sugars in the sample only glucose and fructose were positively identified. A peak, identical with mannitol, was also prominent.

The method has thus not yet been applied successfully to free urinary sugars.

One advantage of the presently described t.l.c. methods over g.l.c. is that each sugar gives a single "spot" and that alditols do not interfere with the revelation of the spots by the sprays described.

G. Paper chromatography of urinary sugars

The introduction of paper chromatographic (p.c.) analysis of monosaccharides by Partridge (1948) not only facilitated their identification but almost immediately enabled their quantitative microdetermination. (Flood et al., 1947; 1948; Hawthorne, 1947).

Paper chromatography has been most frequently employed to detect, separate and measure trace sugars in urine and many relevant papers and reviews (e.g. Menzies & Seakins, 1969) have been published. The following discussion is restricted to applications to urine.

Horrocks & Manning (1949) and Eastham (1949) first examined urinary sugars. Eastham (1949) treated urine with basic lead acetate before chromatography and tentatively identified arabinose, xylose besides glucose and noted the possible presence of rhamnose, as well

as a number of unidentified fast-runners, uronic and ascorbic acids. It was not mentioned under what conditions the urines were collected.

Besides glucose, fructose, mannose, sucrose and several unidentified sugars, Montreuil & Boulanger, (1953) found in non-fasting human urine, spots corresponding to arabinose, xylose and fucose. Rhamnose and galactose occurred in very feeble traces and ribose rarely if at all. Ion exchange resins (Permutit 50 and Amberlite IR 4B) were used to deionise the urine samples.

White & Hess (1956) developed a method for the preparation of a deionised urine concentrate suitable for the detection of small amounts of sugars. Their qualitative two-dimensional paper chromatography (using n-butanol-acetic acid-water and n-butanol-pyridine-benzene-water) on non-fasting urines revealed the presence of spots corresponding to arabinose, xylose, ribose and fucose, besides lactose, galactose, glucose, fructose and glucuronolactone, all detected by aniline-citrate. There were two unknown sugars. Fructose coincided with the arabinose spot. An orcinol-trichloroacetic acid spray showed the presence of sucrose, mannoheptulose, sedoheptulose, fructose, xylulose and ribulose and there were two unknown -uloses. A small amount equivalent to 0.2 ml of original urine was spotted on the paper for chromatography and that ribose and fucose were not always found may have been because too little material was applied to the paper. Like White & Hess (1956) I did not find any definite spot of rhamnose as has been claimed to be present in urine (Bertone, 1963; Montreuil &

Boulanger, 1953). If present, mannose perhaps coincided with the fructose spot. The system of White & Hess (1956) took 48 h to separate the urinary sugars.

By a two-dimensional procedure (using water-saturated phenol, butanol-acetic acid-water), Tower et al. (1956) separated 12 authentic carbohydrate compounds including arabinose, xylose and ribose but not fucose. In numerous deionised samples of 24 h urine (all subjects on a fruit-free diet) eight carbohydrate compounds were detected; arabinose, xylose and ribose besides lactose, glucose, galactose, deoxyribose and a uronide. The presence of fructose was variable; fucose was not reported. The whole operation of one run including the period of drying the chromatogram appeared to take over 72 h.

Apthorp (1956) examined numerous urine samples (both fasting and fed) by descending p.c. (with butanol-acetic acid-water as solvent) and identified glucose in all urines. Lactose was found in urines of all children but only in 60% of the adults. Xylose and arabinose were not always present and their spots partially overlapped; no detail was available about the actual separation of these sugars. The presence of galactose, fructose and sucrose was variable. Neither ribose nor fucose were detected in any one sample, although quantities down to 2.5 µg could be identified. It was not clear whether the spots of the latter sugars separated on the chromatogram.

Howorth & McCredie (1956) and Howorth & MacDonald (1957)by

triangular-shaped p.c. (and with butanol-pyridine solvent and a benzidine reagent developer) examined urines of normal and premature babies. Half of the normals showed xylose with lactose, galactose or a combination of these sugars. Most premature babies showed spots corresponding to xylose, besides glucose, galactose and lactose. No other urinary sugars were detected.

Date (1958a) described a separation and measurement of authentic glucose, galactose, lactose, xylose and arabinose, in 48 h using aniline-citric acid for detection. While examining deionised urines from human subjects he devised (Date 1958b) a two-dimensional procedure. The whole chromatographic operation took 120 h. Sixteen spots (with aniline-citric acid) were detected in deionised urine; of these arabinose, xylose, lactose, galactose and glucose were chromatographically detected and measured. The arabinose and xylose were isolated and respectively identified as the L- and D-enantiomorphs. As will be seen from fig. 1 page 156 (Date, 1958b) the xylose partially overlapped with another (unidentified) spot which from the present work must be presumed to be fucose. These methods were extended to the quantitative estimation of mannose, fucose and ribose (Date, 1958c). Eight years later, Date (1966) reported on measurement of arabinose, xylose, ribose, fucose, lactose and glucose in fasting human urines; his results are discussed in Chapter 6.

As far as I know, Date's is the only p.c. system so far

where anyone has separated and measured in urine all the aldopentoses and fucose. The single greatest criticism of Date's procedure is that it is time-consuming. Moreover, sugar standards can not be run on the same chromatogram with the urine samples. Also, only one urine sample can be spotted on one sheet at a time.

Spots corresponding to arabinose, xylose, lactose, maltose, galactose, glucose, fructose and xylulose in urine were found by Montreuil, (1960). The chromatogram was sprayed both for aldoses and ketoses. Fucose and ribose are not mentioned. Bickel (1961) separated xylose, lactose, galactose, glucose and fructose and examined deionised samples of urine from mature and premature infants and in certain diseases in children. Using detection by aniline phthalate, he found only glucose, galactose, fructose, lactose and sucrose; other sugars were not mentioned. From $R_F$ values of standard sugars on this system fructose was found to overlap with arabinose; fucose was not included in the standards.

Pittera et al. (1963) analysed morning urine samples from 70 subjects. In 15 urines no sugars at all were detected. Only glucose was found in 6 urines, arabinose in 4, and sucrose in 2; both arabinose with glucose were detected in 28. Lactose, galactose, mannose and xylose were detected irregularly. It would appear that the method employed did not give satisfactory separations.

Haller (1965) on wedge-shaped chromatograms sprayed with

aniline phthalate, orcinol-trichloroacetic acid, orcinol-hydrochloric acid and with phloroglucinol, examined deionised fasting urines. Out of 19 subjects he found xylose & glucose in 16, uronic acids in 12, arabinose in 11, galactose in 9, lactose in 8, ribose in 3, sucrose in 2, rhamnose in 2, glucuronolactone in 2, fructose in 2. This work is further discussed in Chapter 6.

Vitek and Vitek (1970) in 42 deionised fasting urines detected 11 known and 3 unknown aldoses, and 8 known and 3 unknown ketoses; sugar spots always corresponded with each of the following in all specimens : arabinose, xylose, ribose, fucose, besides lactose, maltose, galactose, glucose, allose, mannose, N-acetylglucosamine, sucrose, mannoheptulose, sedoheptulose, fructose, alloheptulose, allulose, ribulose and xylulose. Glucuronolactone, rhamnose, deoxyribose and raffinose were variably present. The deionised urine samples were subjected to from four to eight repetitions of one dimensional ascending paper chromatography (83.5 to 217 h) in five solvent systems. Aldoses were detected with aniline-citrate and ketoses with orcinol-trichloro-acetic acid. This paper shows the constancy of composition of the urinary sugars and report the occurrence of allose and alloheptulose. Despite a large amount of urine spotted (10 - 20 min aliquot of diuresis streaked on paper at a time) for chromatography the spots were remarkably compact. Nevertheless Vitek and Vitek's (1970) work was laborious, time-consuming and qualitative. Except arabinose in one system, no other aldopentoses or fucose were separated.

Conclusions on paper chromatography and other above methods applicable to the analysis of sugars in urine:

When appropriate systems are used, paper chromatography is suitable for the separation, detection and quantitation of the numerous free sugars in urine. But with the exception of that of Date (1958a, 1958b, 1958c, 1966) the systems used by other workers were not sufficiently discriminating to permit separation and hence detection of all three aldopentoses and of fucose which both Date and I invariably find to be excreted by normal fasting subjects. Some techniques reported were so insensitive as to fail to detect the commonly accepted urinary sugars such as glucose. Moreover all p.c. especially when done in two-dimensions is time-consuming and therefore not favoured in the routine clinical laboratory.

H. Thin-layer chromatography

Among the adsorbent materials, silica gel, cellulose and Kieselguhr have been more popular than others in the chromatography of sugars.

When the present work was begun I could find no report on quantitative t.l.c. of urinary sugars. The applications of the method by others to the analysis of sugar mixtures especially when arabinose, xylose, ribose and fucose were included is summarised below under headings of the adsorbents used. Some attempts to apply these methods to urine were not satisfactory.

1. Silica gel:

Silica gel, mostly with $CaSO_4$ as binder, has been used most extensively to separate neutral sugars. Developing Silica gel G (Silica gel + $CaSO_4$) with butan-1-ol-acetic acid-diethyl ether-water, Hay et al. (1963) chromatographed various pure monosaccharides but did not achieve separation of ribose, arabinose and xylose. The spots were detected either with concentrated $H_2SO_4$ or alkaline $KMnO_4$. A method (Kaeser & Masera, 1964) which the authors state can be applied to urine or faeces used 0.25 mm layers of Kieselgel G made up with boric acid and developed by propan-2-ol-butan-1-ol-water separated lactose, maltose, sucrose, galactose, glucose, fructose, ribose and palatinose; no results on actual analysis were given. Similarly, Cottee et al. (1964) described the identification of urinary and blood sugars on Kieselgel G with and without borate and with sodium acetate buffer. Separation of a mixture of lactose, maltose, sucrose and fructose took place on the acetate plate and of fructose, galactose, glucose and xylose on borate plate; again no results on urine and blood analysis were given. Arabinose, ribose and fucose were not studied. Satisfactory separation of xylose, arabinose and ribose was not achieved on silica gel buffered with 0.02 M borate. (Jasin & Mishkin 1965).

Ovodov et al. (1967) emphasized that successful sugar separations on buffered silica with $CaSO_4$ depended on the nature and concentration of the impregnating salt; KCL, $NaNO_3$, $K_2SO_4$, KCNS, $Na_2CO_3$, $Na_3PO_4$ and $ZnCl_2$ were suitable. However, some good

separations were obtained using silica made up with sodium salts, the monohydrogen phosphate, the bicarbonate, tungstate, and disodium phenylphosphate. Tables 1 and 4 (Ovodov et al.,1967) show separations of xylose and fucose in two systems. But xylose had an $R_F$ value close to that of arabinose. I used one of the systems of these authors by which they obtained their best results:- 0.5 mm silica gel G layer impregnated with 0.3 M sodium dihydrogen phosphate was developed by butan-1-ol-methanol-water (5:3:1) and the spots were revealed by $H_2SO_4$ - Napthoresorcinol. The sugar spots proved to be well-shaped but there was insufficient spatial separation of fucose from xylose for quantitative work. Ovodov et al. (1967) did not mention the length of their plates. My results were not reproducible perhaps because silica gel was obtained from two different sources.

Unbuffered silica gel G was used to separate pure aldonic acid lactones, aldoses and alditols (Nemec et al.,1967); of the aldoses arabinose, ribose, xylose, glucose, mannose, galactose, rhamnose were run. Arabinose coincided with mannose and xylose with rhamnose.

Lato et al. (1968b) in an intensive work using silica gel (Fluka D.O. without binder) impregnated with 0.03 M $H_3BO_3$, studied many monosaccharides including aldopentoses and fucose in 42 different solvent systems without showing satisfactory separation on unidimensional run. By bidimensional chromatography, both with

20 x 20 cm and 20 x 35 cm plates, as is seen from their chromatographic maps, (fig. 1 - 4, Lato et al., 1968a) 18 sugars were separated including the aldopentoses and fucose. Only 5 µg of each standard sugar was applied; even then the spots were too close to make any quantitative measurement possible. I applied the same system of Lato et al. (1968a) (with Solvent No. 2; page 53 ). With this procedure it was expected that fucose would separate from xylose. The authors' $^{R}F$ values were 36 for xylose and 0.49 for fucose but I found no proper separation of xylose ($^{R}F$ 0.38) from fucose ($^{R}F$ 0.41); again the silica gel was obtained from two different sources. The same authors (1968b) using silica gel with binder (Fluka G) buffered with 0.03 M $H_3Bo_3$ solution on 20 x 35 cm plates obtained only qualitative separation of arabinose, ribose, xylose and fucose. Lato et al. (1969) on silica gel impregnated by either sodium acetate, sodium dihydrogen phosphate or disodium hydrogen phosphate, did not show a single separation between arabinose, xylose, ribose and fucose; a few separations between xylose and fucose were shown but $^{R}F$ differences were small and even in such instances the sugar spots were reported to be diffuse or tailing. In their illustration (fig. 1 page 416; with solvent 36) the only run with both fucose and xylose shows convergence of the fucose and ribose spots; arabinose would partially coincide with ribose ($^{R}F$ values of arabinose and ribose in Table 1 with solvent 36) although in this instance xylose and fucose were clearly separated.

Lato et al. (1968a, 1968b, 1969) used freshly prepared

solution of naphthorescorcinol and $H_2SO_4$ for spraying t.l. plates.

A two-dimensional procedure (Hotta & Kurokawa, 1968) using 0.25 mm thick silica gel F 254 (Merck) separated fucose from other monosaccharides obtained on hydrolysis of glycoproteins, but did not deal with those aldopentoses which occur in association with connective tissue. Kieselgel D.O. with binder, buffered with sodium acetate, was used to separate sugar mixtures containing xylose, fructose, glucose, sucrose, maltose, lactose and raffinose (Stefanis & Ponte, 1968). Triple developments gave good separations of amounts up to 100 µg but ribose, arabinose and xylose were not studied.

Using silica gel on sheets of glass-fibre, buffered with sodium acetate, Kudla & McVean (1968) (development by ethyl acetate-pyridine-water) separated lactose, maltose, sucrose, galactose, glucose, fructose, xylose and ribose after adding authentic sugars to urine. There is no information on fucose and arabinose. The urine (presumably non-fasting) was not deionised.

Conclusions:

It seems that silica gel will separate, and only qualitatively, small amounts ( ~ 5 µg) of a limited number of sugars present in biological fluids. Poor separations of the aldopentoses and of fucose and the low capacity of the plates are disadvantageous in quantitative work.

2. Cellulose:

On cellulose, sugars are separated in systems similar to those used on paper. Most of the location reagents described for paper can be sprayed on to cellulose layers and produce very similar colours. The difficulties of separating fucose on paper are the same as on cellulose layers.

Schweiger (1962) using ethyl acetate-pyridine-water separated galactose, glucose, xylose, ribose and rhamnose but arabinose and mannose coincided and fuoose was not examined. Using 1 mm layers and two solvent systems, Wolfrom et al. (1965) chromatographed a mixture of saccharides, including arabinose, ribose and xylose with similar results. Later Wolfrom et al. (1966) examined, quantitatively, a mixture containing 10 to 50 µg each of glucose, galactose, mannose, xylose and rhamnose. After four developments (5 h) with ethyl-acetate-pyridine-water and spraying with aniline phthalate the coloured sugar spots were extracted with hydrochloric acid in acetone after heating with aniline-phthalate. A linear relationship was found up to 150 µg of each sugar between E and the concentration. The co-efficient of variation obtained of mixtures containing 20 to 100 µg of each sugar was ± 3%. This showed that good quantitative results can be obtained with sugars by means of t.l.c. but arabinose, xylose and fucose were not included. Vomhof & Tucker (1965) examined 9 solvent systems for the separation of aldopentoses, aldohexoses, ketohexoses and some disaccharides. From their $R_F$ values it was clear that not all the

aldopentoses were separated from each other and other sugars in one system. Fucose was not reported. Arabinose, xylose, ribose were separated on cellulose thin-layer in butan-2-one*-acetic acid - saturated aqueous boric acid (Kennedy, 1970). Ersser & Andrew (1970) could not successfully separate three aldopentoses and fucose on cellulose. They detected and separated lactose, sucrose, galactose, glucose, fructose and xylose in acid hydrolysed deionised urine. The method however did not separate arabinose from fructose and fucose from xylose. One µg of the sugar could be detected on the chromatogram but there is no information on the presence of urinary arabinose, fucose and ribose on the chromatogram.

Using the unpleasant mixture of ethyl acetate-pyridine-water (4:3:2) (Bell & Talukder 1970) it was possible on 0.5 mm layer of cellulose (double development) to show separations of aldopentoses which were qualitatively satisfactory but the resolution was not sufficient for quantitative purposes. Fucose coincided with xylose spot.

3. Other thin-layer adsorbent materials of lesser use:

Plaster of paris ($CaSO_4$) was applied by Affonso (1966) to separate (in 30 min) glucose, fructose, mannose, arabinose, xylose and lactose. Applied to urine (Affonso 1967) arabinose, fructose and xylose were tentatively detected. Fucose and ribose were not mentioned.

Other adsorbent materials such as calcium silicate (Jossang,

---

* methyl ethyl ketone

1963), hyflo-super cel and filter cel, (Garbutt, 1964), magnesium silicate (Grasshof, 1964), Alusil (Aluminiumoxide + Silica gel G) (cf. Stahl & Kaltenbach, 1965), polycarbonate (Anderson & Stoddart, 1966) and polyamide layers (Marais, 1967) have been examined for sugar separations without any satisfactory results.

4. Kieselguhr G:

This material (Kieselguhr plus $CaSO_4$) produces a practically inactive retentive thin-layer mainly used for the separation of strongly hydrophilic compounds. Theoretically, it should be possible to fractionate mixtures of sugars with polar solvent systems on inactive thin-layers.

Although later workers have attempted separations of sugars mainly on silica gel, Stahl & Kaltenbach (1961) had for the first time successfully qualitatively separated sugars using 0.25 mm layers of Kieselguhr G buffered with 0.02 M sodium acetate and developed with ethyl acetate-propan-2-ol-water (65:23.5:11.5). The plate (20 x 20 cm) was run for only 10 cm (25 - 30 min) with mixture of 0.5 µg each of lactose, sucrose, glucose, fructose, xylose, ribose, rhamnose and digitoxose. Galactose, fucose and arabinose were not reported upon and this procedure was unsuitable for separation of amounts greater than 1 µg.

De Leenheer and Van Hauwaert (1966) also used Kieselguhr G buffered at pH 5 by 0.1 M phosphate; in deionised urine of unspecified origin, spots were found corresponding to lactose,

galactose, glucose and fructose. These authors did not record the presence of any other sugars; from their methodology it does not seem possible that they could have missed many of the monosaccharides which are frequently detected unless (a) their detection sprays were inefficient or (b) insufficient urine was put on the chromatogram.

In the present work Kieselguhr G has been used as the adsorbent of the t.l.c. for the separation of aldopentoses and fucose. The details of the methodology are described in Chapter 3 and a brief discussion is followed in the next section.

# II DISCUSSION: THE NEW QUANTITATIVE METHODS

## A. Development of thin-layer chromatograms

The successful and rapid one-dimensional procedure described in this thesis resulted from the qualitative observations of Stahl & Kaltenbach (1961) and was arrived at only after many trials and numerous errors. Unsuccessful media proved to be cellulose, silica (alone or buffered), alumina and $CaSO_4$ (appendix II). Only Kieselguhr G suitably impregnated with sodium acetate (slightly basic) or sodium dihydrogen phosphate, could be used to give satisfactory separations of the aldopentoses and fucose from one another and the other sugars of human urine. The original Stahl-Kaltenbach system had to be modified as shown in Table 17.

TABLE 17

Modifications adopted on the Stahl-Kaltenbach (S - K) System

| Conditions | S - K | This thesis |
|---|---|---|
| Wet layer thickness | 0.25 mm | 0.50 mm |
| Developing system (EtOAc - Pr-2-ol - $H_2O$) | 5.65: 2.04: 1 | 4: 2: 1 |
| Length of run | 10 cm | Double development full length of 20 cm plate |
| Molarity of NaOAc | 0.02 | 0.03 |

It is difficult to suggest whether any single factor was responsible for the good spatial separations obtained compared with previously described systems of t.l.c. The thickness of the adsorbent layer may well have contributed; Wolfrom et al. (1965) using cellulose, obtained better separations with layers 1 mm thick than on 0.25 mm ones. The alterations (from S - K) made in the solvent mixture (Table 17) would not be expected to produce any marked change unless the diminution of the proportions of ethyl acetate (the poorest solvent for sugars) and the increase in the relative proportions of propanol and water (in which the sugars will dissolve) is critical in relation to $R_F$ values.

Although a double development separated arabinose from fructose on acetate plates (20 x 20 cm) even 6-fold treatment of 20 x 20 cm plates and 3-fold development of 20 x 35 cm plates failed to separate xylose and fucose. It is known that the distance travelled by sugar spots decreases with the number of developments, more rapidly with faster-moving substances than with slower-moving ones (Halpaap, 1969).

Better separations are known to result from multiple developments either in the same solvent system (Stefanis & Ponte, 1968) or in multiple systems (Vitek & Vitek, 1970). The advantages of multiple development has been discussed by Halpaap (1969).

Ovodov et al. (1967) emphasised that changes in the concentration of the buffering salt in the absorbent layer modify

separations of sugars. When I ran a mixture of monosaccharides on unbuffered Kieselguhr G using the developing solvent given in Table 17 (page 111) all the sugars ran with the solvent front. The role of the buffering salt is not understood - it may be a so-called "inactivation" i.e. reduction in the absorbent properties of the medium or an effect of basicity or acidity. The latter idea is supported by the fact that the hydroxyl groups of sugars and polyols are capable of dissociation at quite markedly different rates. For example, J.C.P. Schwarz (personal communication to Dr. D.J. Bell) who measured dissociation constants ($K \times 10^{14}$) for a number of glyosides and polyols found such differences as Methyl-$\beta$-L-arabinopyranoside (0.67), Methyl-$\alpha$-D-xylopyranoside (1.7) and sucrose (18.7).

It was only on close observation that the urinary xylose spot on an acetate plate sprayed with p-anisidine was found to coincide with the lower three-quarters of another sugar, ultimately detected as fucose. The colour developed by the latter seemed slightly paler than that given by the other authentic sugars. That fucose was indeed the xylose contaminant was shown by its unique chromatographic behaviour when compared with the other 6-deoxyhexoses (page 64 ).

I was therefore confronted with the problem of separating urinary fucose from xylose. Here the work of Ovodov et al. (1967) proved very helpful and led to ultimate success using Kieselguhr G buffered with 0.15 M $NaH_2PO_4$; however, in this system ribose and xylose coincided. Using a plate 35 cm long (fig. 11) authentic xylose and ribose (each 15 µg) could be separated but, when applied to

deionised urine, this separation was inconclusive (fig. 11) possibly because of the presence of polyols (page 66 ). None-the-less, using both acetate and phosphate plates (20 x 20 cm) it became possible to measure, on the former, arabinose and ribose and on the latter fucose and xylose + ribose.

B. Deionisation of urine

The resins used for the present work was derived after White & Hess (1956) who used a mixed bed resin with equal volumes of Amberlite 1R 120 ($H^+$) and 1RA 400 (acetate). Modification has been done (this thesis) because of regeneration difficulty of mixed bed resin.

That the resin columns used for deionisation of urine were efficient, can be said from the recovery study of the aldopentoses and fucose (page 84 to 86 ) and also from the study of Date (1958a, 1958b). In another experiment known amount of urine was passed through six different resin columns (appendix III). After elution and evaporation, spectrophotometry was done for total aldopentoses by the method of Dische & Borenfreund (1958); 85 - 100% recovery was obtained.

Vigorous deionisation was obligatory for good chromatographic result. Cassia & Fertilo (1963) observed that paper strip showed fluorescence from the start to the solvent front if urine was not deionised. This fact has been over and again emphasized, beginning from the first paper chromatographic work of Partridge

(1948). I think that many chromatographic failures or the failure even to detect minor quantities of sugar has been due to poor deionisation of urine. At the begining of the present work I found that if urine was concentrated for the amount of the resins usually used, the separation was poor and a bleached (whitish) background was observed throughout the strip of the run from the origin to the solvent front. However, finally 5 min aliquot of diuresis was found to be suitable for the amount of resin used for this work. Regeneration of a large amount of resin at a time was performed by long columns as described in page 47 . Efficiency of conversion of resins into acetate and $H^+$ form was expected around 85% (BDH, 1965, Ion Exchange Resin). When unwashed IR 120($H^+$) was used, an impurity would cause a whitish spot above the unknown sugar (the sugar first suspected as rhamnose) and make the sugar spot crescent-shaped (fig. 23 ). However this interference was eliminated when the resin was used after washing.

C. Staining methods, extractions and spectrophotometry

1. Staining the sugar spots:

When finding a suitable spray reagent three things were taken into consideration: (1) the reagent must be sensitive and give stable colours with aldopentoses and fucose on the Kieselguhr G buffered plates, (2) the reagent must not give a coloured background, and (3) colours produced must be extractable by a suitable solvent to give a stable solution allowing spectrophotometric measurement,

preferably according to the Beer-Lambert Law. It was found that sprays commonly used on paper, cellulose or silica were generally unsatisfactory on buffered Kieselguhr. Reagents containing aniline salts (Haller, 1965) or salts of 4-aminobenzoic acid with or without sulphosalicylic acid (Bell, 1966) gave very faint colours. Naphthoresorcinol and sulphuric acid (Lato et al., 1968a, 1968b, 1969) gave a poor background. No colour developed after spraying with the acid phloroglucinol reagent of Dische & Borenfreund (1958). Indicators (various pK's) with boric acid failed to show up sugars' colour on the chromatogram. Stable colours, which could be extracted to yield solutions suitable for spectrophotometry were eventually found by use of (i) p-anisidine-sodium dithionite (Pridham, 1956) (Pridham's reagent; page 56) which gave essentially the same brownish colour with all classes of sugars, probably through a kind of Maillard reaction, and (ii) p-aminobenzoic acid-sulphosalicylic acid-$SnCl_2$ (Bell & Talukder, 1971) (PABA reagent; page 54) which differentiated between pentoses and hexoses, probably by dye-formation with furfural or its derivative.

Both the above reagents produced permanent staining of the sugar spots on both acetate and phosphate plates. Background colour of thin-layers remained unchanged for 3 h with (i) and 24 hours with spray reagent (ii).

2. Extraction of the coloured spots:

This is described in the experimental section and no

theoretical explanation can be offered as to why the extractants ultimately used were successful. Methanol and $SnCl_2$ which were successfully used to extract coloured spots given by Pridham's reagent failed to extract colours given by PABA reagent without immediate fading. Acetic acid without stannous chloride failed to retain the extracted colour more than 1 min.

3. Spectrophotometric measurements:

Both the sprays (i) and (ii) described in section C.1. above gave reasonably satisfactory quantitative results (see experimental). Partly because of the difficulty of visual discrimination of colours absorbing visible light around 400 - 370 nm, and partly because of the thickness of the absorbent layer (0.5 mm) "visual inspection" could not be successful with Pridham's spray. The PABA reagent was useful because it visually distinguished different sugar classes; however the extracted colours were not as stable as those generated by Pridham's reagent.

Recently photo-densitometric (surface scanning) determination of the separated sugar spots on t.l. chromatograms have been increasingly popular (Lamkin et al., 1966; Moczar et al., 1967; Kelleher, 1970). These authors found that as little as 0.25 µg of a sugar could be quantitatively determined. There seems to be no theoretical objections to measuring the coloured sugar spots directly on the t.l. chromatograms by surface scanning. Indeed, this would have the advantage of simplicity, sensitivity and quick analysis.

Moreover much thinner layers employing much smaller amounts of applied sugars could be used, and might well have proved more accurate, for scanning, than the thick layers which had to be used in order to obtain sufficient material for conventional spectrophotometry.

# CHAPTER 5

# PRELIMINARY APPLICATION AND RESULTS OF THE NEW METHODS TO MEASURE URINARY ALDOPENTOSES AND FUCOSE

# PRELIMINARY APPLICATION AND RESULTS OF THE NEW METHODS TO MEASURE URINARY ALDOPENTOSES AND FUCOSE

I aimed to investigate the presence and quantities of four free monosaccharides, arabinose, xylose, ribose and fucose in the urine of healthy human subjects and of patients suffering from various diseases. The two original t.l.c. techniques described in this thesis were applied to measure these particular sugars in urine. As a preliminary investigation, three groups of human subjects were examined as follows.

Urine analysis in:-

(1) healthy adults after low and high pentose diet.

(2) healthy fasting subjects.

(3) patients with renal failure.

All urine samples, if they appeared concentrated were suitably diluted to correspond to a standard excretion rate of 1 ml/min. The processing for chromatographic analysis is described in Chapter 3 in detail.

## 1. Urine analysis in three healthy adults after low and high pentose diets

Subjects:

The three subjects were males aged 31, 58 and 66 years of

age not known to suffer from any disease.

(a) Dietary regimens and urine collection

Low pentose/pentosan intake:

The last meal of the previous day was finished by 1900 h. Throughout the experimental period the subjects consumed food ad libitum but were forbidden fruits, fruit juices, vegetables (except potatoes), whole meal flour, beer, cider, whisky, brandy and wine as well as certain items noted in Table 4 (page 40 ). Urines were separately collected during three 8 h periods (2300 to 0700 h, 0700 to 1500 h and 1500 to 2300 h) for two consecutive days. So that two experiments for each period were carried out on each subject. The results of the experiment are given in Table 18.

High pentose/pentosan intake:

From the previous night and throughout the experimental 24 h period, the subjects were allowed food ad libitum to include as much fruit and vegetables as possible; the subjects did not have to record all the pentose/pentosan items consumed or vegetable taken. Urines were collected as above. The results of the experiment are given in Table 19.

(b) Results

Low pentose/pentosan intake (Table 18):

The three subjects in two separate and time divided

xcretion of urinary aldopentoses and fucose on low pentose and pentosan diets; urines were collected over three 8 h periods (A 2300-0700 h; B 0700-1500 h and C 1500-2300 h) for consecutive two days

Rates of excretion, µg/min

| Subjects | Ara | | | Xyl | | | Rib | | | Fuc | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | A | B | C | A | B | C | A | B | C | A | B | C |
| Pas | 22 | 18 | 19 | 11 | 11 | 13 | 6 | 5 | 9 | 28 | 18 | 19 |
| | 16 | 18 | 20 | 10 | 5 | 9 | 8 | 5 | 6 | 28 | 42 | 42 |
| Bel | 20 | 17 | 12 | 10 | 11 | 5 | 6 | 5 | 9 | 8 | 12 | 10 |
| | 11 | 12 | 9 | 9 | 3 | 11 | 8 | 5 | 4 | 13 | 6 | 12 |
| Tal (M) | 12 | 11 | 17 | 7 | 6 | 6 | 5 | 4 | 7 | 12 | 18 | 16 |
| | 7 | 8 | 9 | 6 | 6 | 7 | 3 | 3 | 6 | 9 | 6 | 19 |

experiments showed excretion of the aldopentoses and fucose in regular amounts and no significant differences were found in their rates of excretion when compared with their fasting state. No gross variation was found (except in few instances, e.g. Bell 2nd day's (0700-1500 h) xylose value and Tal (M) 2nd day's (0700-1500 h) fucose value) between the three 8 h periods and it appeared that there was no obvious circadian rhythm in the excretion of these sugars.

High-pentose intake (Table 19):

In most of these experiments, both arabinose and xylose showed enhanced rates of excretion; excretion rates of xylose were most enhanced. It is important to note that in no instance were the rates of ribose and fucose noticeably altered from excretion rates of those shown during fasting.

Urine analysis in healthy fasting volunteers

Subjects:

Six males and six females, clinically healthy, took part. Four males and six females were of ages between 19 and 31 years. Two of the males were of 58 and 66 years of age.

Dietary regimens and urine collection:

All the volunteers fasted overnight for 12 h; their last meal was finished by 1900 h. Items of food as mentioned under the

TABLE 19

Excretion of urinary aldopentoses and fucose by subjects on high pentose/pentosan diet.

Urines were collected over three 8 h periods

(A, 2300-0700; B, 0700-1500 h and C, 1500-2300 h) for consecutive two days

Rates of excretion μg/min

| Subjects | Ara | | | Xyl | | | Rib | | | Fuc | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | A | B | C | A | B | C | A | B | C | A | B | C |
| Pas | 27 | 37 | 35 | 13 | 52 | 26 | 7 | 10 | 16 | 22 | 36 | 30 |
| | 10 | 15 | 26 | 15 | 35 | 50 | 4 | 3 | 3 | 23 | 33 | 22 |
| Bel | 20 | 12 | 34 | 61 | 39 | 46 | 4 | 4 | 9 | 17 | 8 | 10 |
| | 30 | 34 | 31 | 41 | 35 | 32 | 6 | 5 | 6 | 9 | 10 | 15 |
| Tal (M) | 11 | 38 | 43 | 6 | 152 | 127 | 3 | 4 | 3 | 7 | 8 | 8 |
| | 21 | 16 | 14 | 110 | 218 | 115 | 8 | 6 | 3 | 14 | 17 | 16 |

regimen of low pentose/pentosan diet were prohibited. Any urine passed before 2300 h was discarded. Three male and three females rejected their first morning samples and immediately drank as much water as they could tolerate and subsequently collected a sample of urine before breakfast. Measured and times urine samples collected on two successive days were subjected to the analytical procedure as described in Chapter 3.

Results:

The results (Tables 20 and 21) include those of two successive morning samples from each of the three male adults on the low pentose/pentosan diet, collected under the conditions specified as 'fasting'.

TABLE 20

Rates of excretion of aldopentoses and fucose (µg/min) in urines of 6 healthy fasting males (on 2 separate days)

| Subjects | Ara | Xyl | Rib | Fuc |
|---|---|---|---|---|
| Pas | 22 | 11 | 6 | 28 |
| | 16 | 10 | 8 | 28 |
| Bel | 20 | 10 | 7 | 8 |
| | 11 | 9 | 4 | 13 |
| Tal (M) | 12 | 7 | 5 | 12 |
| | 7 | 6 | 5 | 9 |
| McA | 11 | 7 | 5 | 17 |
| | 17 | 7 | 5 | 16 |
| McN | 14 | 6 | 6 | 14 |
| | 12 | 6 | 8 | 14 |
| Pal | 13 | 10 | 4 | 16 |
| | 12 | 10 | 4 | 26 |
| Range | 7 - 22 | 6 - 11 | 4 - 8 | 8 - 28 |
| Mean ± SD | 14 ± 4.1 | 8 ± 1.9 | 5.5 ± 1.4 | 17 ± 6.9 |

TABLE 21

Rates (μg/min) of excretion of aldopentoses and fucose in urines of 6 healthy fasting female subjects (on 2 separate days)

| Subjects | Ara | Xyl | Rib | Fuc |
|---|---|---|---|---|
| Tal (F) | 10 | 11 | 3 | 14 |
| | 12 | 10 | 4 | 18 |
| McK | 13 | 6 | 3 | 15 |
| | 16 | 10 | 8 | 23 |
| Cal | 14 | 9 | 6 | 15 |
| | 16 | 11 | 5 | 22 |
| Cur | 16 | 6 | 4 | 7 |
| | 11 | 11 | 4 | 19 |
| Law | 11 | 9 | 5 | 14 |
| | 9 | 9 | 4 | 16 |
| Nay | 10 | 6 | 3 | 17 |
| | 13 | 8 | 4 | 18 |
| Range | 9 - 16 | 6 - 11 | 3 - 8 | 7 - 22 |
| Mean ± SD | 13 ± 2.5 | 9 ± 1.9 | 4.5 ± 1.4 | 16 ± 4.1 |

It is found that each 12 h fasting urine sample (Table 20 & 21) always contain aldopentoses and fucose. There is no significant difference in excretion rates between the corresponding sugars in two sexes ($P > 0.05$). Photographic representation of the fasting urinary sugars from t.l.c. are reproduced in fig. 9, 10 & 11.

Trace urinary sugar in 6 subjects with renal failure

Subjects and urine collections:

Two consecutive 24 h urine samples were collected from each of five hospitalised patients with some form of renal failure. Two were diagnosed as suffering from acute-on-chronic glomerulonephritis, one from uraemia after polyarteritis nodosa and the other two from acute tubular necrosis. During the period of urine collection the creatinine clearance of these patients ranged from 1 - 34 ml/min. They were all on intermittent dialysis, on a low protein, low sodium and low potassium diet and received glucose as their main caloric source. These patients were oliguric except one (uraemia following polyarteritis nodosa). The sixth subject, an ambulant, mature university student, had suffered from chronic renal failure and hypertension for several years and had been on antihypertensive and diuretic drugs. His urine samples were collected over two consecutive 24 h periods. He was on a diet containing 40 - 50 g of protein and was instructed to avoid high pentose/pentosan foods during the period of urine collection.

The clinical data of these patients are given in brief in Table 22.

Urine deproteinisation:

All samples of urine from renal failure subjects contained mild to moderately heavy amounts of protein; this had to be removed before subsequent quantitative chromatography. To avoid unnecessary

TABLE 22

Clinical data of the 6 patients with renal failure

| Name | Sex & Age in Years | Diagnosis | Blood urea mg % | Creatinine clearance ml/min | Treatment | Diet (Na & K restricted) | Subsequent course |
|---|---|---|---|---|---|---|---|
| Low | f 54 | Polyarteritis nodosa with uraemia | 262 | 25 - 34 | Peritoneal dialysis steroids | 20 - 50 g protein | alive with chronic renal failure. |
| Bro | f 23 | Chronic glomerulo-nephritis | 420 | 1 - 2 | Haemodialysis | 20 - 50 g protein | after kidney transplant, died of graft rejection. |
| Ait | m 67 | Carcinoma of bronchus Myocardial infarction Acute tubular necrosis | 287 | - | Peritoneal dialysis | 20 - 40 g protein | died of acute tubular necrosis. |
| Mur | m 50 | Chronic renal failure due to hypertension | 60 | - | Haemodialysis | 50 g protein | died of viral hepatitis. |
| Gom | m 61 | Acute tubular necrosis | 380 | 10 | Haemodialysis | 20 - 40 g protein | alive with chronic renal failure |
| Bar | m 37 | Chronic renal failure | 194 | 23 | Antihypertensives Diuretics (later on inter-mittent haemodialysis). | 40 g protein (only Na restricted) | died of septicaemia. |

dilution, a measured amount of urine was well mixed with one-hundredth of its volume of saturated $Fe_2(SO_4)_3$ solution and an excess of solid calcium carbonate rapidly added (Bell, 1970). After mixing at $40^{\circ}$, and removal of solids by centrifugation, a clear neutral solution was obtained. This deproteinised urine, in amounts equivalent to 10 min excretion was then deionised for chromatography.

Rechromatography:

In all the six subjects the urinary "glucose" was so great (clinitest $\frac{1}{4}$ - $\frac{1}{2}$%) as to occupy a large area on the subsequently developed chromatograms. While on an acetate plate this did not interfere with the separation and measurement of arabinose and ribose (fig. 22), on phosphate-plates, streaking of glucose interfered with the separation of the combined xylose + ribose spot and then of fucose. Rechromatography of these samples were done as follows:- an acetate-plate was developed but not sprayed as usual containing a 5 min excreted urine. Guided by an additional plate, simultaneously developed, but sprayed, an area of the Kieselguhr corresponding to the spots of arabinose, xylose + fucose and ribose was removed from the unsprayed plate and the solid extracted 4 times with 25 ml amounts of 80% aqueous methanol. The eluate was filtered, evaporated and rechromatographed on a phosphate plate as before. The recoveries of xylose by this process in 3 experiments were 77, 92 and 90% (mean 86%).

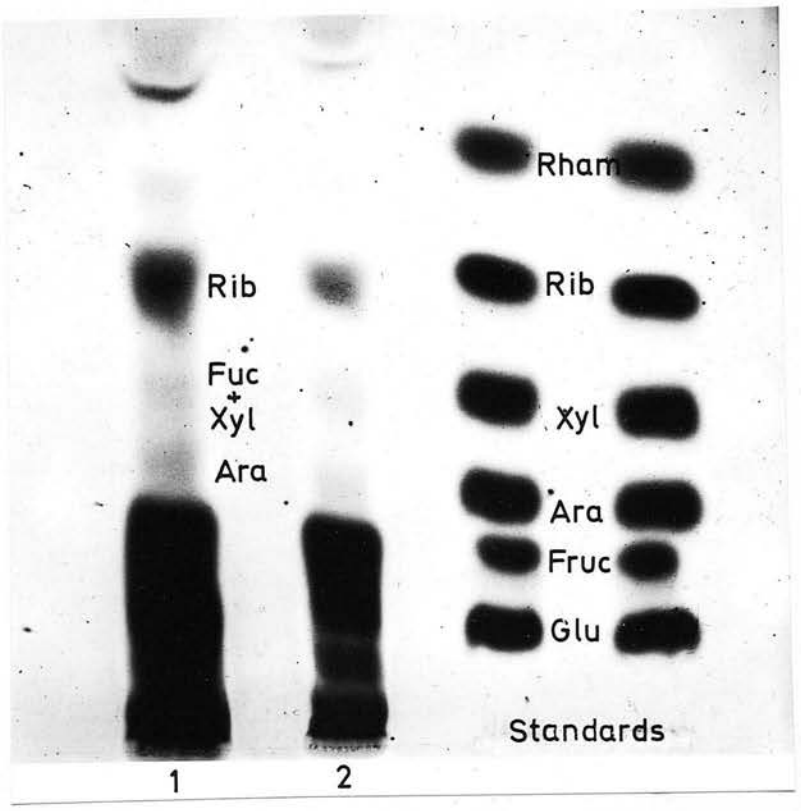



Fig. 22. T.l.c. on acetate plate (20 x 20 cm) after double development showing extent of separation of authentic (standard) sugars (15 ug each) and of urinary sugars of 1 min (strip 1) and $\frac{1}{2}$ min (strip 2) excretion volumes from a patient of renal failure.

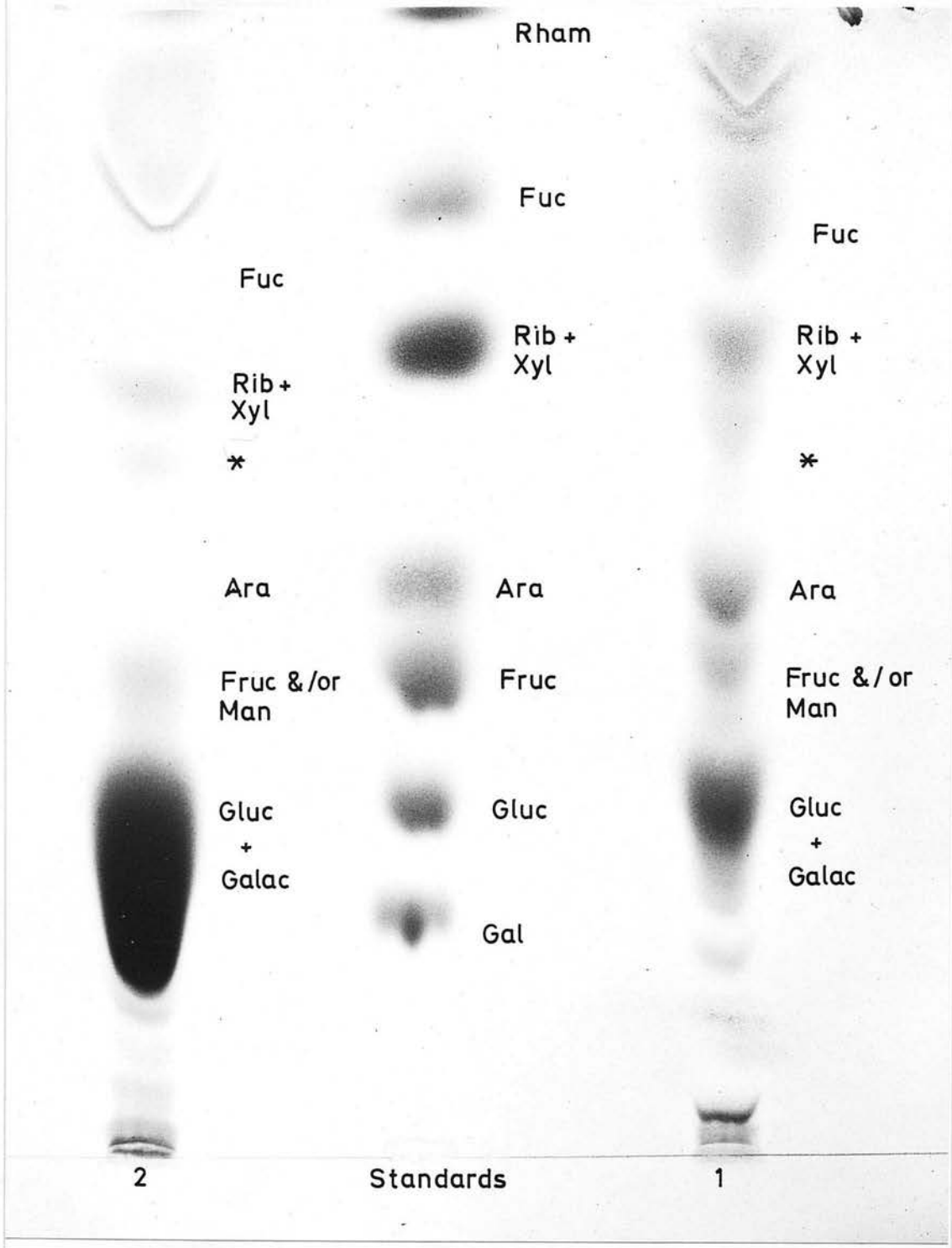
Note: Urinary arabinose, xylose+fucose spots are much less intense than that of ribose. Arabinose and ribose were measured without rechromatography (see text, page 129).

Fig. 23. T.l.c. on phosphate plate (20 x 20 cm) after double development showing extent of separation of authentic (standard) sugars (15 µg each) and of urinary sugars.

Strip: 1. in urine volume of $\frac{1}{2}$ min excretion from a normal fasting individual.

Strip: 2. in a rechromatographed urine sample from a patient of renal failure (1 min excretion volume).

Note: absence of fucose and trace amount of ribose+ xylose in Strip 2.




(Fig. 23. See opposite page)

Preliminary qualitative t.l. chromatograms showed that these patients with renal failure excreted very little aldopentose and fucose. To achieve reasonable spectrophotometric extinctions, urine volume corresponding to 3 and 5 min excretion were spotted on both acetate and phosphate plates respectively.

Results:

Table 23 shows that the excretion of the aldopentoses and fucose in the 6 cases of renal failure is a close similarity. Figs. 22 and 23 show chromatogram of a urine sample from a renal failure patient.

TABLE 23

Rates of excretion of four urinary sugars

by six patients of renal failure

The cipher 0 indicates "below limits of detection"

| Subjects | 24 h urine volume in ml | Urine protein (boiling test) | Urine glucose (clini test) | µg/min | | | |
|---|---|---|---|---|---|---|---|
| | | | | Ara | Xyl | Rib | Fuc |
| Lov | 1470 | + | ¼ | 3 | 0 | 3 | 0 |
| | 2220 | + | ½ | 1 | 0 | 4 | 0 |
| Bro | 345 | + | ¼ | 2 | 1 | 3 | 3 |
| | 260 | + | ¼ | 1 | 1 | 3 | 2 |
| Mur | 46 | + | ¼ | 4 | 2 | 6 | 1 |
| | 50 | + | ¼ | 3 | 2 | 4 | 1 |
| Gom | 610 | + | ¼ | 2 | 0 | 4 | 4 |
| | 380 | + | ¼ | 1 | 0 | 2 | 2 |
| Ait | 78 | + + | ¼ | 3 | 2 | 5 | 0 |
| | 30 | + + | ¼ | 2 | 2 | 5 | 0 |
| Bar | 2035 | + + | ¼ | 2 | 1 | 3 | 0 |
| | 1905 | + + | ½ | 2 | 1 | 3 | 0 |
| | | Ranges | | 1 - 4 | 1 - 2 | 2 - 6 | 0 - 4 |
| | | Mean | | 2.2 | 1.1 | 3.7 | 1.1 |

When compared with normals P is $< 0.001$ for arabinose, xylose and fucose but $< 0.02$ and $> 0.01$ for ribose.

# CHAPTER 6

## I. DISCUSSION OF RESULTS

## II. POSSIBLE FUTURE APPLICATIONS OF T.L.C. INVESTIGATIONS

# DISCUSSION OF RESULTS

## Qualitative chromatography

All the fasting normal subjects showed similar patterns of monosaccharides in their urines; using both acetate and phosphate plates, arabinose, xylose, ribose, fucose, maltose, lactose, sucrose, fructose and/or mannose, glucose and galactose were invariably present and each sugar apparently occurred in closely similar amounts. On the acetate plates, five, and on phosphate plates, seven so far unidentified sugar "spots" could be observed, (fig. 12 and 13). The following 6-deoxyhexoses were eliminated from the "unknowns": the 6-deoxy analogues of D-allose, D-glucose, D-gulose, L-idose and D-talose. A "spot" closely similar to 6-deoxy-mannose (rhamnose) was invariably present but this component cannot yet be positively identified. As unknown sugars, allose and N-acetyl glucosamine could not be excluded; Vitek & Vitek (1970) found evidence for their presence. In addition, no evidence was found for the presence in urine of: allulose, apiose, lyxose, sorbose, sedoheptulose, mannoheptulose and erythrose. The four last-named sugars gave no detectable colours with Pridham's or PABA spray reagents on both acetate and phosphate plates.

Since my aim was to measure aldopentoses and fucose, no further effort was made to detect ketoses on acetate and phosphate plates with reagents known to be specific for them (e.g. Klevstrand & Nordal, 1950; Dedonder, 1952).

Several workers have reported human urines to contain numerous sugars (for references, see Bell, 1967). The excretion of several urinary sugars is known to be influenced by food (White & Hess, 1956; Date, 1958b). To know whether a urinary sugar has an endogenous origin, the urine must be collected in the post-absorbtive state. Several workers have examined urine for sugars under fasting conditions; those detected are most likely to have endogenous sources. (Table 24).

Sugar excretion patterns of non-fasting subjects who have been on diets low in pentose / pentosans may be mentioned here. In 3 such subjects I found an excretion pattern identical with those of fasting normals. Tower et al. (1956) in non-fasting humans consuming diets free from fruits and vegetables, qualitatively detected lactose, galactose, glucose, arabinose, xylose, ribose, 2-deoxy-ribose, a uronide and, occasionally, fructose in their urines. White & Hess (1956) in a single subject on the same regimen, found lactose, galactose, glucose, fructose, arabinose, xylose, fucose, ribose, glucuronolactone, sucrose, sedoheptulose, xylulose and ribulose. However, they did not always detect ribose and fucose even when their subjects consumed a mixed diet. By the technique of one-dimensional paper chromatography, on 19 subjects fasting overnight, Haller (1965) found no xylose in 3, no arabinose in 5 and no ribose in 16. Date (1966), Vitek & Vitek (1970) and the present author invariably find all three aldopentoses and fucose in urine from fasting healthy subjects. Haller's failures to detect

TABLE 24

Trace sugars detected in fasting human urine

| Authors | Method | Spraying reagent | Fasting urinary sugars detected |
|---|---|---|---|
| Apthorp (1956) | Paper Chromatography | Aniline hydrogen phthalate; naphtho-resorcinol. | Lac, Suc, Gal, Glc, Fru, Ara and Xyl. |
| Date (1958b, 1966) | Paper Chromatography | Aniline citrate | Lac, Gal, Glc, Ara, Xyl, Rib, Fuc. |
| Haller (1965) | Paper Chromatography | Aniline phthalate; orcinol-HCl ; phlorglucinol. | Gal, Glc, Fru, Ara, Xyl, Rib, Rha, glucuronolactone. |
| Vitek & Vitek (1970) | Paper Chromatography | Aniline citrate; orcinol-trichloro acetic acid. | Lac, Mal, Gal, Glu, All, Man, Ara, GlcNAc, Xyl, Fuc, Rib, glucuronolactone, Suc, mannoheptulose, sedoheptulose, Fru, alloheptulose, allulose, xylulose, and ribulose. |
| This thesis | Thin-layer Chromatography | Pridham's; & PABA | Lac, Mal, Suc, Gal, Glc, Fru and/or mannose, Ara, Xyl, Rib, Fuc. |

these sugars are probably due to using a method of insufficient sensitivity. Apthorp (1956) detected neither ribose nor fucose in "fasting" urines; this may have been due to inadequate deionisation, to the application of insufficient material to his chromatograms and/or to the difficulty of separating fucose from xylose.

In my systems, fructose and mannose coincided on the chromatograms but it may be presumed, if Vitek's (1970) findings are substantiated, that both sugars are normally present. In general, failures to detect every neutral sugar which may occur in "fasting" urine may be due to (1) incomplete deionisation, (2) application to the chromatogram of an insufficient quantity of material, (3) failure to achieve chromatographic separation and (4) insensitive spray-reagents. When collecting urine from fasting subjects care must be taken to ensure that no foods which would act as sources of sugars have been consumed for several hours before the fast commences.

The quite separate techniques employed by Vitek & Vitek (1970) and by the present author show that the excretion patterns of those neutral sugars, which both have identified, are similar.

Quantitative aspects

Measurements were made of the rates of excretion of the four sugars under investigation in 12 fasting healthy adults on 2 successive days. Although the results are insufficient to show how such values might differ significantly from one day to another, there is no reason to believe that they do so during fasting; any

differences which were observed were within the experimental error. (See coefficient of variation, page 84 ).

In the group of fasting adults, 2 elderly persons (Bel and Pas) were included; both took part in the dietary experiment. No variation with age in the excretion rates of the sugars in question was found by Date (1966). Moreover, elderly persons do not differ from younger ones in their capacity to absorb monosaccharides (Cori, 1925). Although the experimental numbers were small, Bel and Pas did not differ from the younger subjects except that Pas repeatedly excreted fucose at a higher rate but this may have been due to the small number of samples and may eventually prove to be within a normal but more extended range.

In agreement with Date (1966) the present study showed no significant sex difference ($P > 0.05$) between 6 males and 6 females (Tables 20 & 21).

Arabinose always predominated among the fasting urinary aldopentoses, and ribose was excreted at the lowest rate. Tables 20 and 21 show that the excretion rates of these sugars are in the approximate ratios of arabinose, 1.0; xylose, 0.6; ribose, 0.4. Tower *et al.* (1956) "semiquantitatively" assessed in one subject on a fruit-free diet, the excretion rate of 13, 4 and 6 µg/min and the ratios to be 1: 0.3: 0.5. Date (1966) found "quantitative" values corresponding to 1.0; 0.4: 0.25. Haller's (1965) quantitative results are related to urine volume and not to time, in fasting

samples and show a wide scatter, e.g. Ara, 16 - 62 μg/ml; Xyl, 31 - 100 μg/ml and Rib, 8 - 32 μg/ml; these figures, if they can at all be compared with Date's findings and those of this thesis, would suggest a different series of proportions.

Previous measurements on fasting human urine of the three aldopentoses and fucose have been made only by Date (1958b, 1966). He examined (1958b) two samples of urine from one fasting subject and measured (and identified) L-arabinose and D-xylose besides glucose, galactose and lactose; ribose and fucose were not mentioned. Subsequently Date (1966) examined the urine of 4 subjects who had fasted for three 4 h periods subsequent to a previous fast of 8 h. Date's results are given in Table 25.

TABLE 25

Excretion rates of the aldopentoses and fucose by four fasting adults during three 4 h periods subsequent to a previous 8 h fast

(Modified from Date, 1966 Table 1)

| Urinary sugars | Excretion μg/min/1.73 $mm^2$ body surface ± S.D. | | |
|---|---|---|---|
| | 8 - 12 h | 12 - 16 h | 16 - 20 h |
| Ara | 17 ± 6 | 15 ± 3 | 15 ± 3 |
| Xyl | 11 ± 8 | 7 ± 1 | 5 ± 1 |
| Rib | 4 ± 2 | 5 ± 1 | 5 ± 1 |
| Fuc | 15 ± 7 | 12 ± 4 | 13 ± 4 |

The table shows that Date found no appreciable change in the rates of excretion of these sugars. My findings agree well withhis results(on three subjects consuming food of a low pentose/ pentosan content Table 18) which indicated no evidence of a circadian rhythm of aldopentose excretion, nor of fucose excretion.

Date (1966) analysed urine samples for aldopentoses, fucose, lactose and glucose from 6 fasting men and 8 fasting women of whom 8 were young (20 - 32 yrs.) and 6 were older subjects (46 - 71 yrs.). The results are given in Table 26 (except the values for lactose and glucose).

TABLE 26

Excretion rates (μg/min/1.73 $m^2$) of aldopentoses and fucose in urines of 8 young, 6 older and 6 male, 8 female healthy subjects (young and old were selected from the 14 male and female subjects).

| Sugars | Young | Older | Females | Males |
|---|---|---|---|---|
| Ara | $17 \pm 4$ | $17 \pm 5$ | $16 \pm 4$ | $19 \pm 4$ |
| Xyl | $7 \pm 2$ | $7 \pm 2$ | $6 \pm 1$ | $7 \pm 2$ |
| Rib | $4 \pm 1.5$ | $4 \pm 1.5$ | $4 \pm 1.4$ | $4 \pm 1.5$ |
| Fuc | $13 \pm 5$ | $14 \pm 5$ | $12 \pm 5$ | $16 \pm 5$ |

No significant difference ('t' - test) between any groups.

In the present study the urine samples from 6 males and 6 females were analysed (Table 20 and 21). In agreement with Date (1966) no significant sex difference was found. For comparison, the quantitative data obtained by Date (1966) and the present author is summarised in Table 27.

TABLE 27

Quantitative data on urinary aldopentoses

| Author | Method | Subject(s) | Excretion µg/min | | | |
|---|---|---|---|---|---|---|
| | | | Ara | Xyl | Rib | Fuc |
| Date (1966) | Quantitative Paper Chromatography | 14 fasting | 17 ± 4 | 7 ± 2 | 4 ± 1.5 | 13 ± 5 |
| Present author | Quantitative t.l.c. | 12 fasting | 13 ± 3 | 8 ± 2 | 5 ± 1.5 | 17 ± 6 |

As will be seen, considering the experimental difficulties involved in quantitative chromatography, the two sets of data are in reasonable agreement, even when it is remembered that my xylose figures are arrived at by difference. It would be unwise to compare the above results with those assessed by Tower et al. (1956) on a single subject and with those of Haller (1965) who did not always detect allthe aldopentoses in his samples from 19 fasting subjects. Moreover, Haller's results are widely different (pages 138 & 139).

"Apparent" total aldopentose and true aldopentose value compared

At the beginning of the present work it was proposed to

measure initially the total urinary aldopentoses ("apparent" total aldopentose) on the samples those would be subjected to chromatographic analysis. In fact the necessity to be able to measure individual aldopentoses developed from the significantly discordant observations of different workers (see introduction and Section II of this chapter). In collaboration with Drs. D. J. Bell and R. E. Cull (Bell et al., 1972; the paper is appended to the thesis) many analyses were made, by the phloroglucinol method of Dische & Borenfreund, (1957) including ones on the 21 urine samples of 11 fasting subjects which were also analysed for individual aldopentoses. While it is not intended to include here details of these experiments, some of the relevant results are brought into discussion here.

A comparison of direct analyses by phloroglucinol and by t.l.c. of deionised urine for total aldopentose (Bell et al., 1972 Table 1) shows that the first method averages about 7 μg/min higher than the amounts found by chromatography (Table 28).

The average "apparent" total aldopentose by phloroglucinol in 9 samples of 5 male subjects is 34 ± 6.4 μg per min and true aldopentose by t.l.c. is 27 ± 6 μg/min; in 12 samples of 6 female subjects they are 34 ± 10.9 μg/min and 27 ± 3.6 μg/min respectively. Urine deionisation eliminates glucuronic acid, glucuronosides, sugar phosphates and as well as bases and unknown factors which interfere with the measurement of true total aldopentoses.

TABLE 28

Comparison of direct analyses by chemical methods and by t.l.c. of deionised urine (µg/min)

| Authors | Subjects | "Apparent" total urinary aldopentoses | True total urinary aldopentoses |
|---|---|---|---|
| Tower *et al.* (1956) | 8 adults on fruit free diet | 23 ± 4.8 | - |
| | 1 adult on fruit free diet | - | 23 |
| Date (1966) | 14 adults male and female | - | 28 ± 7.5 |
| Bell *et al.* (1972) and this thesis | 5 adult male (9 samples) | 34 ± 6.4 | 27 ± 6 |
| | 6 adult female (12 samples) | 34 ± 10.9 | 27 ± 3.6 |

The difference between the "apparent" total aldopentose value obtained by Tower *et al.* (1956) and Bell *et al.* (1972) is most probably due to different methods of assessment but their true urinary total aldopentose values are much closer.

There was no significant sex difference in the "apparent" total urinary aldopentoses by the phloroglucinol technique although three females gave values unusually high (Bell *et al.*, 1972 Table 1). Date's (1966) values on separate measurements of arabinose, ribose

and xylose for 14 male and female (Table 26 this thesis) can be collected as total aldopentose and is included in Table 28 for comparison.

Alimentary problems

i) Low pentose/pentosan intake:

The measurements on three subjects on a low pentose/pentosan intake are given in Table 19. As numbers are small no statistical analysis has been done; differences, if any, between the rates observed in the three 8 h consecutive periods and those obtained on the fasting subjects are obviously slight. Whether items of food consumed by these subjects on the pentose/pentosan-free diet contained any pentose other than ribose is not known. No increased rate of excretion of any of the sugars under examination was detected. This experiment yielded the following information: (1) during each consecutive 8 h period the excretion rate of each sugar appeared approximately constant, (2) no rate differed appreciably from those seen after fasting and (3) hence to determine the endogenous excretion rates of aldopentoses and of fucose it is only necessary to ensure that the subject has consumed no pentose/pentosan rich food for 12 h and that the urine specimen is timed.

ii) Alimentary pentosuria:

After a high pentose/pentosan diet the excretion rates of both arabinose and xylose in urine were enhanced in all the three subjects (Table 20) especially in the instance of xylose.

Date (1958b) obtained similar results, xylose being excreted in highest amounts and then arabinose. In no subject was any change, compared with their fasting rates, noted with regard to ribose and fucose; it would thus seem that items of food do not detectably contribute to their excretion. Alimentary pentosuria was first described by Johnstone (1906) but its nature was not revealed until the analyses made by Date (1958b) which are confirmed by the present work especially that alimentary pentosuria is mainly due to xylose. The amounts of xylose and arabinose in urine reflect the ingested proportions of these free sugars.

The results obtained in 3 subjects after low pentose/pentosan and high pentose/pentosan foods are summarised in Table 29.

TABLE 29

Rates of excretion of aldopentoses and fucose (μg/min) over 24 h by 3 subjects on low pentose/pentosan and high pentose/pentosan intakes. Each figure represents the average of 6 different samples analysed for 2 consecutive days over three different 24 h periods.

| Subjects | Pentose intake | Ara | Xyl | Rib | Fuc |
|---|---|---|---|---|---|
| Pas | Low | 19 | 10 | 6 | 30 |
| | High | 25 | 32 | 5 | 28 |
| Bel | Low | 14 | 8 | 5 | 10 |
| | High | 27 | 42 | 6 | 12 |
| Tal (M) | Low | 11 | 7 | 5 | 14 |
| | High | 24 | 121 | 5 | 12 |

It may be noted from Table 29 that arabinose and xylose excretion enhanced in each subject but not ribose and fucose. These results are discussed in view of their known metabolic disposition (see below).

iii) Do pentose-containing hemicelluloses contribute to human carbohydrate metabolism?

As yet there is no evidence that aldopentoses when combined in dietary macromolecules can contribute to the nutritional status of man. None-the-less, ingested pentosans which are loosely termed hemicelluloses and contain arabofuranose and xylopyranose units, among others, do disappear largely during their passage through the human alimentary tract presumably through the activities of the micro-organisms (McCance & Laurence, 1929; Southgate & Durnin 1970). Selliere (1908, 1909) and Swartz (1910-11) found strong evidence for (bacterial) xylanase activity in the human colon. Whether free pentoses, unutilised by the liberating organisms, ever diffuse across the wall of the large intestine and thus might contribute to the "endogenous" excretion of urinary pentoses is not known. The <u>in vitro</u> study of Southgate (1965) shows that massive oxidation of the sugar units liberated in the large intestine by the enteric flora takes place and therefore a colonic contribution to the urinary pentoses is most unlikely. It is theoretically possible that the arabinose units of "hemicelluloses" all of which are furanosidic, could undergo hydrolytic liberation through the action of gastric HCl at $37^{\circ}$. However there is no evidence known to confirm this.

The "fasting" excretion of aldopentoses and fucose is time-dependent.

That these sugars are excreted independently of urine volume was apparent from the results of the experiments involving low-pentose/pentosan diets (Table 19). This was also observed by Date (1966) (Table 25 in this thesis), and confirms the more elementary experiments on total aldopentose excretion described by Bell et al. (1972) involving water-diuresis. These results are strongly in favour of an endogenous origin for the sugars in question, especially if one takes into account the sources of experimental errors in this type of work. It may be noted that Keller (1965) found that in water-diuresis during fasting, the rate of endogenous glucose excretion is also related to time.

Aldopentose and fucose metabolism in man and how these free sugars might arise endogenously in human tissue:

Since the discovery of alimentary pentosuria by Johnstone (1906), the misconception has been prevalent that aldopentoses (xylose especially) are not metabolised by mammals. Current views (e.g. Latham et al.,1971) seem unaware of work which goes back 40 years when it was well established that oral D-xylose could be metabolised by normal and diabetic humans and dogs. (Marble & Strieck, 1932; Grafe & Reinwein, 1932; Bassler et al.,1965) (See also chapter 2). Less than half of the sugar appeared in the urine. Twenty five years later Wyngaarden et al. (1957) showed that 60% of intravenously infused D-xylose, L and D-arabinoses, D-lyxose and D-ribose were all utilised by man; up to 40% of the given dose was

excreted in urine.

In my subjects with a high pentose/pentosan diet there were increase in rates of urinary excretion of arabinose and xylose. The reason why only a proportion of D-xylose and L-arabinose enters into the metabolic pathways, is not known. Renal excretion can be accounted for by postulating a low rate of tubular reabsorption.

Certain possibilities can be considered regarding the unchanged rate of excretion of ribose and fucose. Apparently free ribose occurs in much smaller amounts in our daily food in comparison to xylose and arabinose. The fate of the ribose consumed is not definitely known. It might be set free during digestion, and carried by the circulation to where it is metabolised. The utilisation of D-ribose by the mammalian body was shown by Segal & Foley (1958) to be much more complete than that of L-arabinose and D-xylose. D-ribose is metabolised by human erythrocytes but not D-xylose and L-arabinose (Lachhein & Matthies, 1960). The average total urinary excretion of ribose after a 15 min infusion was 21% of the dose. A much smaller amount was excreted when the sugar was infused at a constant but slower rate.

Free L-fucose is not known in foods; it is, however, widely distributed in combination in very many tissue components of animals, plants and bacteria (cf. Ishihara et al., 1968). The α-L-fucosidase occurs (Bocci & Winzler, 1969) in rats' intestines. If mammalian intestines in general possess this enzyme some L-fucose will be

assimilated from foods. However, free somatic L-fucose could arise in two ways, (see also Chapter 2); (a) from ingested food, or (b) by intrasomatic synthesis from glucose. The somatic availability of free fucose arising from its enzymic hydrolytic liberation in the small intestine is speculative. But a single study on the human metabolism of L-fucose is known to the writer; Segal & Topper (1960) injected, I.V. 5 μCi (1- $^{14}C$) of the sugar into a healthy adult; its metabolic disposition showed that 30% was excreted in urine (Chapter 2 page 30). Thus it could be expected that, if much dietary fucose was available, some would appear in the urine; this was not so. So there were three possibilities, (1) that the human intestine lacked α-L-fucosidase activity so that bound α-L-fucose units were not there set free, (2) that no absorption of free fucose took place and (3) that any fucose absorbed was fully utilised by the body.

The present results and those of Date (1958b, 1966) show that the urinary aldopentoses and fucose occur in regular amounts, per healthy individual, after a fast of at least 12 h duration; these sugars are therefore of endogenous origins and will be excreted under any healthy circumstance. The somatic sources of these sugars can only be speculative. A number of studies (Table 2 page 13) have shown that L-arabinose, D-xylose and L-fucose are structural components of certain *GGG and peptidoglycans. D-xylose and L-arabinose serve as points of linkage between carbohydrate and protein in these mammalian tissue components.

---

* peptidoglycosaminoglycuronoglycans

But it is not known whether katabolism of the complex molecules results in liberation into the blood stream of the aldopentoses (and fucose) so that, in some way, they escape somatic utilisation and thus subsequently appear in the urine. Little can be said about the liberation of bound xylose either *in vivo* or *in vitro* and nothing about bound arabinose. Lysosomal β-D-xylosidase was found to have only weak activity *in vitro* against the β-D-xylosyl link to serine (Fisher *et al.* 1966).

L-fucose is widely distributed as a component of many proteoglycans (Table 2) especially in those secreted by epithelioid tissues, in oligosaccharides of human milk and in blood group substances. The L-fucopyranosyl residues contribute to the specificity-determining structure of the H, $Le^a$ and $Le^b$ blood-group substances (Watkins, 1966). It is not known whether combined fucose can be hydrolysed off so that it could contribute to the urinary component. But in contrast to xylose and arabinose, much evidence shows the possibility that combined fucose could be liberated at various sites in the animal body (Chapter 2)

Mammalian tissues contain L-α-fucosidase activity which could liberate fucose from milk oligosaccharides (Levvy and McAllan, 1961). Its absence of activity leads to the deposition of fucose-rich glycoproteins causing 'fucosidosis'(Van Hoof & Hers 1968; Durand *et al.*, 1969). The porcine kidney enzyme can split off fucose from blood group substances A & H (Vidershayn & Rosenfeld 1969a). Rats gastrointestinal tract appears to contain

L-$\alpha$-fucosidase (Bocci & Winzler 1969). Such evidence suggests that either (1) free fucose is continuously produced and, carried by the plasma to appear in the glomerular filtrate and that renal excretion results from incomplete tubular reabsorption; or (2) kidney basement membrane (Dische et al., 1968) which itself contains fucose-proteoglycans, is continuously attacked by L-$\alpha$-fucosidase and is the source of urinary fucose.

D-ribose in RNA, in ribonucleotides and ribonucleosides is of course a universal cellular component. The presence of free ribose apart from in urine has been reported in beef, lamb and pork muscle (Macy et al., 1964) and in human skin (Jacobi, 1969). This sugar, ofcourse, is liberated from RNA through the successive activities of ribonuclease, nucleotidase and nucleosidase. But it must be said that we really know little about the extent of these enzymic activities.

In a preliminary investigation, after deproteinising, deionising and then applying an equivalent of 5 ml of fasting human plasma on the acetate plate, I found traces of free arabinose, xylose and ribose. Free fucose was detected, through g.l.c. in human plasma by Horning & Horning (1970). The presence in plasma of these free sugars suggests that they may either arise by katabolism of their host-molecules or that they are intermediates in anabolism or have arisen by dephosphorylation from aldopentose phosphates associated with the HMP. Fucose, likewise may occur as a result of katabolic or anabolic mechanisms.

It is unlikely that the small amounts of aldopentoses and of fucose in urine are introduced from the post-tubular urinary tract, because all the six patients with renal failure (Chapter 5 page 133) excreted only ribose at the lower end of the normal rate and xylose, arabinose and fucose only in traces or not at all. The renal basement membranes cannot be ruled out as origin of the free sugars. There is indirect evidence that the sugars might originate from these sources. First, bound fucose has been detected in the glomerular basement membrane and although xylose and arabinose have not been looked for in the GGG of kidney glomerulus and tubules, their presence there is not unlikely. Bound ribose is invariably present in all cells. Secondly, highly active α-L-fucosidase occurs in renal tissue (pigs). A renal origin for urinary arabinose, fucose and xylose is supported by the results on the renal-failure patients mentioned above and might be related to the proportion of intact nephrons. Incomplete tubular reabsorption is undoubted when aldopentoses are administered in gram quantities either I.V. or by mouth. Grafe & Reinwein (1932), Marble & Strieck (1932) and Bassler et al. (1965) have all shown the capacity of humans and dogs to metabolise D-xylose given orally where about 40% of the dose appeared in the urine. Wyngaarden et al. (1957) administered to man, intravenously, D-xylose and L-arabinose (and certain other aldopentoses) and observed again that about 40% of the dose was excreted irrespective of the sugar given. Segal & Foley (1958) after I.V. infusion of D-ribose found about 20% of the dose to be excreted. It is still obscure why these low molecular weight

sugars suffer from incomplete reabsorption.

Shanon (1938) studied the renal handling of xylose in dogs and found that experimental elevation of the plasma glucose to 300 mg/100 ml saturated the renal glucose-reabsorbing mechanism, with a concomitant (but reversible) inhibition of the reabsorption of xylose. Shanon (1938) and Smith (1951) concluded that xylose is reabsorbed by an active tubular process identical with that responsible for the reabsorption of glucose, as it is completely blocked by phlorizin. The glucose - xylose reabsorption mechanism is always working in favour of the much greater proportion of glucose in the glomerular filtrate so that "active sites" within this mechanism may seldom be free to deal with xylose. There is no information on the renal handling of arabinose.

In contrast to the increased excretion of xylose, diabetics with blood glucose concentrations around 300 mg/100 ml excreted no greater amounts of ribose than did subjects with normal blood sugars, at the gram level of the experimental observations (Segal & Foley, 1958). On the other hand, when fasting diabetics were examined by Date (1966), increased rates of excretion of ribose were observed. Such differences between the physiological - pathological levels of tissue components and experiments where there is massive administrations of sugars (and other substances), must always be borne in mind when interpreting results in terms of the metabolic capacity of an animal or its organs or tissues.

Nothing is known about the renal handling of fucose.

Thus from the above discussion it appears that the origin of these sugars remains unsolved.

Renal failure

At least under conditions of starvation, the human kidney plays a gluconeogenetic role (Owen et al., 1969). Analyses of the urines of patients suffering from renal failure was also stimulated by the observation (Krebs & Lund, 1966) that rat kidney cortex preparations are very active in forming glucose from D-xylose and L-arabinose but not from D-ribose or L-fucose; the rates of conversion to glucose of other sugars in the kidney cortex are of the same order of magnitude as those observed in liver preparations. Since Date (1966) showed that, in hepatic cirrhosis, the urinary excretion rates of arabinose, xylose and fucose were significantly increased, it was considered that somewhat similar results might be observed in patients suffering from failures of renal function. On the contrary, the observations on the six patients showed the exact opposite. Each patient with renal failure excreted extremely low amounts of arabinose, xylose and fucose. (Healthy adults vs. renal patients, $P < 0.001$). On the other hand the rate of excretion of ribose was not appreciably diminished and was at the lower extremity of the normal range ($P < 0.02 > 0.01$). This suggests that in renal failure ribose may appear in the urine because it originates in some metabolic pathway separate from those concerned with the other sugars under examination.

While glucosuria was noted in each renal patient, no suggestion can be offered to account for the low/absent rates of excretion of arabinose, xylose and fucose. Forty years ago Fishberg & Friedfeld (1932) proposed, as a result of interesting experiments, that the excretion of fed D-xylose could be used as an index of human renal function. Although the analytical methods then employed would not now be considered entirely acceptable, they showed that the assessed xylose excreted by diseased kidneys was about one tenth of that eliminated in health. Moreover, in place of the steady fall in the "xylose" blood-level seen in health, in renal failure it remained steady for at least 10 h. The results were considered to be due to the failure of the damaged kidneys to concentrate xylose by more than 0.1 - 0.2%. Normal kidneys were shown to be able to concentrate xylose to 2.5% during two h. The loss in concentrating ability by a damaged kidney was attributed by Fishberg & Friedfeld to have a correlation with a diminution in the number of functional nephrons.

The present observations on the low rates of excretion of arabinose, xylose and fucose in renal failure may thus be due to a lack of functional nephrons. These questions could be partly or even completely answered if the levels of these sugars would be measured in plasma, and would substantiate the Fishberg-Friedfeld proposition if the plasma concentration of these sugars were high in cases of renal failure. From the results obtained on the cases of renal failure two further possibilities can be put forward.

(1) If the nephrons themselves produce these sugars, in renal failure their excretion would be at rates proportional to the number of intact nephrons. Practically, the excretion rates of these sugars in patients with renal failure appears to correlate with their rate of glomerular filtration (1 - 34 ml/min). Whatever might be the origin of these sugars it seems very probable that excretion of xylose, arabinose and fucose is related to the number of intact nephrons.

(2) Diseased renal cortical tissue might be stimulated to remove, from the plasma, larger amounts of arabinose, xylose and fucose than it does in health, as an aspect of its gluconeogenetic capacity (cf. Krebs & Lund, 1966). While such a suggestion might well, on cursory inspection seem to be unlikely, it must always be remembered that we really know surprisingly little (despite the volume of current research) about what is actually going on within that extraordinary natural object, the human body.

## II. POSSIBLE FUTURE APPLICATIONS OF TLC INVESTIGATIONS.

The present knowledge on the metabolism of free aldopentoses and fucose in human body is limited and the origin of these sugars is still obscure. Understanding of the metabolism of these sugars will be clearer when these have been measured in urine and plasma of subjects suffering from various diseases. Because it is possible rapidly and completely to separate and measure so many sugars (mono- and disaccharides) by the new chromatographic procedures described in this thesis, investigations of human metabolic pathways other than glucose should be facilitated. The relatively enormous proportion of the blood glucose interferes (as has been seen with urinary glucose in renal failure patients, - this thesis page 129 ) with the chromatography of other trace sugars. Measurements of non-glucose sugars will be possible (personal communication from Dr. D. J. Bell) after removal of blood glucose by washed suspension of yeast (Somogyi, 1927). This avoids the cumbersome method of rechromatography (page 129 ). Thus the new t.l.c. techniques which provide separations of galactose and fructose might well prove helpful in metabolic errors involving these sugars.

The whole range of connective tissue disorders might be worth looking at; since xylose, arabinose and fucose are components of GGG and proteoglycans some deviations from normal in the value of these sugars in urine and in plasma may be likely. Date's (1966) results of the urinary sugars in patients suffering from diabetes

mellitus, infectious diseases, cirrhosis of liver, hyperthyroidism and coronary occlusion need elaboration and confirmation.

An investigation of aldopentose excretion rates in hyperthyroidism might be made. Coover et al. (1950) claimed that rats' urine contained significantly raised total aldopentose after exposure to low ambient temperatures and when given thyroid extracts. Further, thyroid feeding markedly increased and thiouracil reduced the output of urinary pentose (Roe & Coover, 1950). In severe craniofacial injury, Masturzo & Negro (1953) found high aldopentose levels in their patients' plasma and urine. The individual aldopentose value in these conditions may further the understanding of the pathogenesis of these disorders.

There is a possibility that some varieties of muscular dystrophy may result in ribosuria (pages 6 & 8). This has never been proved. For the determination of ribose on the acetate plate, 3 min excreted urine should be applied on the chromatogram as with one min's volume the sugar was estimated at the lowest level of sensitivity.

Fucose estimations in plasma and urine may be done in varieties of conditions. 'Fucosidosis' (Van Hoof & Hers, 1968; Durand et al., 1969) may be associated with total absence of free fucose in plasma and in urine. Plasma protein bound fucose has been estimated in many pathological conditions (Sharma & Sur, 1969; Marshall et al., 1970), but nothing is known on the value of this sugar in free form.

The new t.l.c. techniques can be applied for the analysis of any food item for aldopentoses, fucose and other sugars.

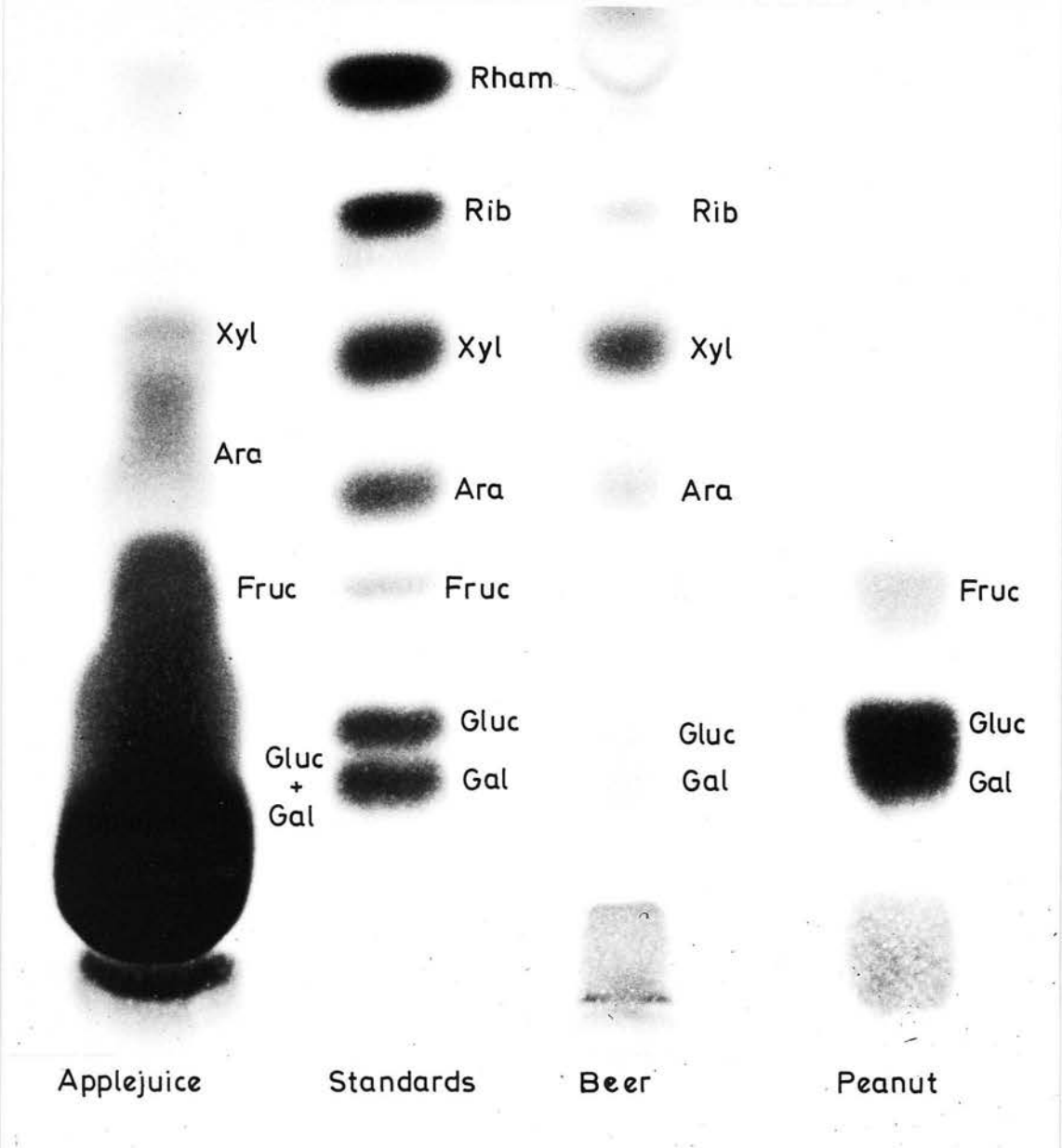



Fig. 24. T.l.c. of apple-juice, beer and peanuts on acetate plate (20 x 20 cm) after double development and spraying with PABA reagent.

On acetate plate I chromatographed (purely qualitative) a sample of apple juice and beer after deionisation and peanuts after fat extraction and deionisation. Apple juice contained free arabinose and xylose; beer, - arabinose, xylose and ribose. Peanuts did not show up any of these free sugars. None of the above contained fucose (fig. 24).

Different mammalian tissues can be looked for free sugars or after hydrolysis. The knowledge of the occurrence of these sugars in tissues will add to the understanding of the metabolism of the sugars.

# BIBLIOGRAPHY

BIBLIOGRAPHY


Affonso, A. (1966). J. Chromatogr. 21, 332.

Affonso, A. (1967). J. Chromatogr. 27, 324.

Agranoff, B.W., and Brady, R.O. (1956). J. Biol. Chem. 219, 221.

Anderson, D.H.W. and Stoddart, J.F. (1966). Carbohydrate Res. 1, 417.

Apthorp, G.H. (1957). J. Clin. Path. 10, 84.

Arwidi, B. and Samuelson, O. (1964). Anal. Chim. Acta. 31, 462.
Chem. Abstr. (1965). 62, 721 g.

Aso, K., Watanabe, T., Sasaki, K., and Motomura, Y. (1961).
Tohoku J. Agr. Res. 12, 261.
Chem. Abstr. (1962). 56, 15965 e.

Aspinall, G.O., Begbie, R., and McKay, J.E. (1967).
Cereal Science Today 12, No. 6.

Bahl, O.P. (1969). J. Biol. Chem. 243, 575.

Bassler, K.H., Holzmann, H., Prellwitz, W. and Stechert, J. (1965).
Klin. Wschr. 43, 27.

Bell, D.J. (1962). In Comparative Biochemistry, edited by Florkin, M. and Mason, H.S., Vol. III. Chapter 7, p. 287.
Academic Press. New York. London.

Bell, D.J. (1966). J. Chem. Soc. C. p. 1638.

Bell, D.J. (1967). Comp. Biochem. Physiol. 20, 523.

Bell, D.J. (1970). Q. Jl. exp. Physiol. 55, 59.

Bell, D.J., Cull, R.E., and Talukder, M.Q.-K. (1972).
Int. J. Biochem. 3, 33.

Bell, D.J., and Talukder, M.Q.-K. (1970). J. Chromatogr. 49, 469.

Bell, D.J., and Talukder, M.Q.-K. (1971). Biochem. J. 122, 24p.

Bertone, E. (1963). Minerva Med. 54, 975.

Bhatti, T. and Clamp, J.R. (1968). Clin. Chim. Acta. 22, 563.

Bickel, H. (1961). J. Pediat. 59, 641.

Bierman, E.L., Baker, E.M., and Plough, I.C. (1959).
Diabetes. 8, 455.

Bocci, V., and Winzler, R.J. (1969). Am. J. Physiol. 216, 1337.

Bossman, H.B., Hagopian, A., Eylar, E.H. (1968).
Arch. Biochem. Biophys. 128, 1968.

Bourne, E.J., Foster, A.B., and Grant, P.M. (1956).
J. Chem. Soc. p. 4311.

Bray, H.G., Henry, H., and Stacey, M. (1946). Biochem. J. 40, 124.

Bray, B.A., Lieberman, R., and Meyer, K. (1967).
J. Biol. Chem. 242, 3373.

Brien, F.S., Turner, D.A., Watson, E.M., and Geddes, J.H. (1952).
Gastroenterology. 20, 287.

Brink, N.G. (1953). Acta Chem. Scandinav. 7, 1081.

Bucolo, G., and Bartlett, G.R., (1960). Biochem. Biophys. Research Communs. 3, 620.

Burns, J.J., and Evans, C. (1956). J. Biol. Chem. 223, 897.

Caldwell, R.C., and Pigman, W. (1966). Arch. Oral Biol. 11, 437.

Carlson, D.M. (1968). J. Biol. Chem. 243, 616.

Carlson, D.M. and Hansen, R.G. (1961). Federation Proc. 20, 84.

Carubelli, R. (1966). Carbohydrate Res. 2, 480.

Cassia, B. and Fertilo, S. (1963). Boll. Soc. Ital. Biol. Sper. 39, 335. Chem. Abstr. (1964). 60, 4438 c.

Charles, J. (1962). Compt. Rend. 255, 761.

Chester, M.A., and Watkins, W.M. (1969). Biochem. Biophys. Res. Communs. 34, 835.

Chumbalov, T.K., and Nurgaliera, G.M. (1966). Khim. Prirodn. Soedin, Akad. Nauk. USSR. 2, 284.
Chem. Abstr. (1966). 65, 20509 a.

Churms, S.C. (1970). In Advances in Carbohydrate Chemistry and Biochemistry 25, 13.

Clamp, J.R., Bhatti, T. and Chambers, R.E. (1971).
Methods of Biochemical Analyses, edited by Glick, D. 19, 229.

Clamp, J.R., and Putman, F.W. (1964). J. Biol. Chem. 239, 3233.

Coffey, J.W., Miller, O.N., and Sellinger, O.Z. (1964). J. Biol. Chem. 239, 4011.

Conchie, J., and Hay, A.J. (1963). Biochem. J. 87, 354.

Consden, R., and Stanier, W.M. (1952). Nature. 169, 783.

Coover, M.O., Feinberg, L.J., and Roe, J.H. (1950). Proc. Soc. Exper. Biol. & Med. 74, 146.

Cori, C.F. (1925). J. Biol. Chem. 66, 691.

Cottee, J., Mathieu, M., and Collombel, C. (1964). Pathol. Biol. Semaine. Hop. 747. Chem. Abstr. (1964) 61, 13619 d.

Cuatrecasas, P., and Segal, S. (1966). J. Biol. Chem. 241, 5910.

Date, J.W. (1958a). Scandinav. J. Clin. & Lab. Invest. 10, 149.

Date, J.W. (1958b). Scandinav. J. Clin. & Lab. Invest. 10, 155.

Date, J.W. (1958c). Scandinav. J. Clin. & Lab. Invest. 10, 444.

Date, J.W. (1966). Scandinav. J. Clin. &Lab. Invest. 18, 45.

Davidson, S., and Passmore, R. (1969). Human Nutrition and Dietetics, 4th Edition. p. 14. E. & S. Livingstone Ltd. Edinburgh and London.

Dawson, G. and Clamp, J.R. (1970). Methods in Medical Research 12, 131.

Dedonder, R. (1952). Bull. Soc. Chim. biol. Paris, 34, 144.

De Leenheer, A., and Hauwaert, van. M. (1966). Pharm. Tijdschr. Belg. 43, 97. Chem. Abstr. (1966). 65, 15778 a.

D'Elia, R., and Zacchello, F. (1968). Boll. Soc. Ital. Biol. Sper. 44, 1957. Chem. Abstr. (1969). 71, 36938 S.

Demanur, R., Fauconneau, G., and Guntz, G. (1958). Compt. Rend. 246, 2820.

Dische, R.M., Pappas, G.D., Grauer, A., and Dische, Z. (1965). Biochem. Biophys. Res. Communs. 20, 63.

Dische, Z. (1962a). In Methods in Carbohydrate Chemistry, edited by Whistler, R.L. and Wolfrom, M.L. Vol. I p. 487, Academic Press. New York and London.

Dische, Z. (1963). Ann. New Y. Acad. Sci. 106, 259.

Dische, Z., and Borenfreund, E. (1957). Biochem. Biophys. Acta. 23, 639.

Dische, Z., and Zelmenis, G. (1964). Federation Proc. 23, 319.

Drew, A.L., and Selving, B.T. (1953). Neurology. 3, 563.

Durand, P., Borrone, C. and Della Cella, G. (1969). J. Pediat. 75, 665.

Eastham, M. (1949). Biochem. J. 45, xiii.

Efron, M.L. (1968). in Chromatographic & Electrophoretic Techniques, edited by Smith, I. Volume II p. 184. William Heinemann. Medical Books Ltd.

Ersser, R.S. and Andrew, B.C. (1971). Medical Lab. Technology. 28, 355.

Esan, P., and Amerine, M.A. (1966). Amer. J. Enol. Viticult. 17, 265. Chem. Abstr. (1967). 66, 74914 k.

Farshtchi, D., and Moss, C.W. (1969). J. Chromatogr. 42, 108.

Fishberg, E.H., and Friedfeld, L. (1932). J. Clin. Invest. 11, 501.

Fisher, D., Higham, M., Kent, P.W., and Pritchard, D. (1966). Biochem. J. 98, 46 p.

Flood, H.E., Hirst, E.L., and Jones, J.K.N. (1947). Nature. 160, 86.

Flood, H.E., Hirst, E.L., and Jones, J.K.N. (1948). J. Chem. Soc. p 1679.

Foster, D.W., and Ginsburg, V. (1961). Biochim. Biophys. Acta. 54, 376.

Fourman, L.P.R. (1948). Clin. Sci. 6, 289.

Fransson, L-A. (1968). Biochim. Biophys. Acta. 156, 311.

Fredholm, H. (1960). Acta Chem. Scandinav. 14, 437.

Garbutt, J.L. (1964). J. Chromatogr. 15, 90.

Gardell, S. (1953). Acta Chem. Scandinav. 7, 201.

Garrod, A.E. (1909). Inborn Errors of Metabolism p. 82. Reprinted with a supplement by H. Harris. London : Oxford University Press, 1963.

Ghalamber, M.A. and Heath, E.C. (1962). J. Biol. Chem. 237, 2427.

Gibbons, R.A. (1959). Biochem. J. 73, 209.

Gibbons, R.A. and Roberts, G.P. (1963). Ann. N.Y. Acad. Sci. 106, 218.

Ginsburg, V. (1958). J. Am. Chem. Soc. 80, 4426.

Ginsburg, V. (1960). J. Biol. Chem. 235, 2196.

Ginsburg, V. (1961). J. Biol. Chem. 236, 2389.

Ginsburg, V., and Kirkman, H.N. (1958). J. Am. Chem. Soc. 80, 3481.

Ginsburg, V., and Neufeld, E.F. (1969). Annual Review of Biochemistry. 38, 371.

Grafe, E., and Reinwein, H. (1932). Dt. Arch. Klin. Med. 173, 646.

Grasshof, H. (1964). J. Chromatogr. 14, 513.

Grau, R., Gunther, H., and Scheper, J. (1960). Fleischwirtschaft 9, 728.

Green, M., and Cohen, S.S. (1956). J. Biol. Chem. 219, 557.

Greenwald, I. (1930). J. Biol. Chem. 88, 1 & 89, 501.

Gregory, J.D., Laurent, T.C. & Roden, L. (1964). J. Biol. Chem. 239, 3312.

Grollman, A.P., Hall, C.W. and Ginsburg, V. (1965). J. Biol. Chem. 240, 975.

Grollman, A.P., and Marcus, D.M. (1966). Biochem. Biophys. Res. Communs. 25, 542.

Gross, D. (1953). Nature. 172, 908.

Haller, H. (1965). Med. Welt. 206 and 258.

Halpaap, H. (1969). In Chromatographic and Electrophoretic Techniques, edited by Smith, I. Vol. 1. 3rd Edition. p 835. William Heinemann. Medical Books Ltd.

Harding, V.J., Nicholson, T.F., and Archibald, R.M. (1936). Biochem. J. 30, 326.

Harding, V.J., and Selby, D.L. (1931). Biochem. J. 25, 1815.

Hashimoto, Y., Mori, I., and Kimura, M. (1952). Nature. 170, 975.

Hawthorne, J.R. (1947). Nature. 160, 714.

Hay, G.W., Lewis, B.A., and Smith, F. (1963). J. Chromatogr. 11, 479.

Hay, G.W. and Smith, F. (1962). Am. Soc. Brewing Chemists Proc. 127. Chem. Abstr. (1964). 60, 12633 a.

Hayman, S., and Kinoshita, J.H. (1965). J. Biol. Chem. 133, 503.

Heath, E.C., and Ghalamber, M.A. (1962). J. Biol. Chem. 237, 2423.

Heath, E.C., and Roseman, S. (1958). J. Biol. Chem. 230, 511.

Helting, T. and Roden, L. (1968). Biochem. Biophys. Acta 170, 301.

Hers. H.G. (1956). Biochim. Biophys. Acta. 22, 202.

Heteryi, Jr. G. and Ishiwata, K. (1968). Am. J. Physiol. 214, 1333.

Heyningen, R. van. (1958). Biochem. J. 69, 481.

Heyningen, R. van. (1959). Biochem. J. 73, 197.

Hiatt, H.H. (1957). J. Clin. Invest. 36, 1408.

Hiatt, H.H., and Lareau, J. (1960). J. Biol. Chem. 235, 1241.

Hickman, J., and Ashwell, G. (1956). J. Amer. Chem. Soc. 78, 6209.

Hollman, S., and Touster, O. (1956). J. Am. Chem. Soc. 78, 3544.

Hollman, S., and Touster, O. (1957). J. Biol. Chem. 225, 87.

Hoof, van., Hers, H.G. (1968). Lancet, 1, 1198.

Horecker, B.L. (1969). In Pentoses and Pentitols. edited by Horecker, B.L., Lang, K. and Takagi, Y. p.5. Springer-Verlag. Berlin. Heidelberg. New York.

Horning, E.C., and Horning, M.G. (1970). Methods in Medical Research, 12, 400.

Horrocks, R.H., and Manning, G.B. (1949). Lancet, 1, 1042.

Hotta, K., and Kurokawa, M. (1968). Anal. Biochem. 26, 472.

Hough, L., Jones, J.K.N., and Wadman, W.H. (1949). J. Chem. Soc. p 2511

Howorth, J.C. and MacDonald, M.S. (1957). Arch. Dis. Child. 32, 417.

Howorth, J.C., and McCredie, D. (1956). Arch. Dis. Child. 31, 189.

Huang, P.C. and Miller, O.N. (1958). J. Biol. Chem. 231, 201.

Hughes, R.C. and Whelan, W.J. (1958). Chemistry and Industry p. 884.

Inoue, S., and Yosizawa, Z. (1966). Arch. Biochim. & Biophys. 117, 257.

Ishihara, H., and Heath, E.C. (1968). J. Biol. Chem. 243, 1110.

Ishihara, H., Massaro, D.J., and Heath, E.C. (1968). J. Biol. Chem. 243, 1103.

Jacin, H., and Mishkin, A.R. (1965). J. Chromatogr. 18, 170.

Jaffee, W.G. (1962). Acta Cient. Venezolana 13, 100. Chem. Abstr. (1963). 58, 1669 f.

Jacobi, O. (1969). Arch. Klin. Exp. Dermatol. 233, 383. Chem. Abstr. (1969). 70, 85450 q.

Jeanloz, R.W. (1964). Medicine. 43, 363.

Jelenkova, K., Kubicek, R., and Santavy, F. (1961). Sb. Praci. Prir. Fak. Palack. Univ. Olomuci. p. 67.

Johnstone, R.W. (1906). Edinburgh Med. J. 20, 138.

Jolley, R.L. and Freeman, M.L. (1968). Clin. Chem. 14, 538.

Jolley, R.L., Warren, K.J., Scott, C.D., Jamchill, J.L. and Freeman, M.L. (1970). Amer. J. Clin. Path. 53, 793.

Jones, N.R. (1958). J. Sci. Food Agric. 9, 672.

Jonsson, P., and Samuelson, O. (1967a). Anal Chem. 39, 1156.

Jonsson, P., and Samuelson, O. (1967b). J. Chromatogr. 26, 194.

Jossang, P.T. (1963). J. Chromatogr. 12, 413.

Kaesar, H., and Masera, G. (1964). Schweiz. Med. Wochschr. 94, 158.
Chem. Abstr. (1965). 62, 13497 c.

Katsura, N., and Davidson, E.A. (1965). Federation Proc. 24, 60.

Kaufman, R.L., and Ginsburg, V. (1968). Exptl. Cell. Res. 50, 127.

Kawamura, S., and Narasaki, T. (1959). Bull. Agr. Chem. Soc. Japan.
23, 471. Chem. Abstr. (1959). 54, 16553 h.

Kawamura, S., and Narasaki, T. (1961). Agr. Biol. Chem. 25, 527.

Keith, N.M., Power, M.H., and Peterson, R.D. (1934).
Proc. Staff Meet Mayo Clinic, 9, 43.

Keller, D.M. (1965). Clin. Chem. 11, 471.

Kennedy, J.F. (1970). Chromatographia. 3, 316.

Kent, P.W. (1962). Gastroenterology. 43, 292.

Kent, P.W. (1967). In Essays in Biochemistry, edited by Campbell,
P.N. and Greville, G.D. Vol 3, p. 105. Academic Press.
London. New York.

Kent, P.W. and Mah, G.K. (1961). In proceedings of the fifth
international congress of biochemistry, Moscow, 1961, edited
by Sissakian, N.M. Volume IX Plenary sessions and abstract
of papers. Pergamon Press, Oxford, London, New York, Paris.

Kent, P.W., and Marsden, J.C. (1963). Biochem. J. 87, 38 p.

Kent, P.W., Molineux, I.J., Haegele, M.R. and Stevenson, F.K. (1969). Biochem. J. 114, 87 p.

Kesler, R.B. (1967). Anal. Chem. 39, 1416.

Khym, J.X. and Zill, L.P. (1952). J. Am. Chem. Soc. 74, 2090.

King, H.K. (1942). Biochem. J. 36, 43.

Klevstrand, R. and Nordal, A. (1950). Acta Chem. Scandinav. 4, 1320.

Knecht, J., Cifonelli, J.A., Dorfman, A. (1967). J. Biol. Chem. 242, 4652.

Koscielak, J. (1963). Biochim. Biophys. Acta. 78, 313.

Kositkovskya, O.M., and Rosenfeld, E.L. (1962). Biokh. 27, 912.

Krebs, H.A., and Lund, P. (1966). Biochem. J. 98, 210.

Kudla, R.M., and McVean, D.E. (1968). Am. J. Clin. Pathol. 49, 279.

Kuhn, R. (1958). Bull. Soc. Chim. Biol. 40, 297.

Lachhein, L., Grube, E., Johnigk, C., and Matthies, H. (1961). Klin. Wochschr. 39, 875.

Lachhein, L., and Matthies, H. (1960). Acta Biol. Med. Ger. 4, 403. Chem. Abstr. (1960). 54, 21392 d.

Lamkin, W.M., Ward, D.N. and Walborg, Jr. E.F. (1966). Anal. Biochem. 17, 485.

Lang, K. (1969). Pentoses and Pentitols, edited by Horecker, B.L., Lang, K. and Takagi, Y. Springer-Verlag Berlin. Heidelberg. New York, 1969.

Larsson, L.-I., and Samuelson, O. (1965). Acta Chem. Scand. 19, 1357.

Lasker, M., Enklewitz, M., and Lasker, G.W. (1936). Human Biol. 8, 243.

Latham, M.C., Abraham, J., Ramos, C., Mora, O., Romero, E., Cobos, F. (1971). Lancet, 2, 405.

Lato, M., Brunelli, B., Ciuffini, G., and Mezzetti, T. (1968a). J. Chromatogr. 34, 26.

Lato, M., Brunelli, B., Ciuffini, G., and Mezzetti, T. (1968b). J. Chromatogr. 36, 191.

Lato, M., Brunelli, B., Ciuffini, G., and Mezzetti, T. (1969). J. Chromatogr. 39, 407.

Lehnhardt, W.F. and Winzler, R.J. (1968). J. Chromatogr. 34, 471.

Letzig, E., and Nuernberger, H. (1963). Nahrung. 7, 518. Chem. Abstr. (1963). 59, 14501 c.

Levvy, G.A., and McAllan, A. (1961). Biochem. J. 80, 435.

Lichev, V., and Panaiotov, I.M. (1958). Bulgar. Akad. Nauk. Izvest. Khim. Inst. 167. Chem. Abstr. (1960). 54, 817 f.

Lilyblade, A.L., and Peterson, D.W. (1962). J. Food Sci. 27, 245.

Lindahl, U. (1966). Biochim. Biophys. Acta. 130, 361.

Lindahl, U., and Roden, L. (1965). J. Biol. Chem. 240, 2821.

Macleod, A.M. and Preece, I.A. (1953). J. Inst. Brew. 60, 1954.

Macy, Jr. R.L., Naumann, H.D., and Bailey, M.E. (1964). J. Food Sci. 29, 136.

Malpress, F.H., and Hytten, F.E. (1958). Biochem. J. 68, 708.

Mandel, I.D. and Ellison, S.A. (1962). Ann. N.Y. Acad. Sci. 106, 271.

Marais, J.P. (1967). J. Chromatogr. 27, 321.

Marble, A., and Strieck, F. (1932). Dt. Arch. Klin. Med. 173, 538.

Marby, C.C., Gryboski, J.D., and Karam, E.A. (1963). J. Lab. Clin. Med. 62, 817.

Marks, P.A., and Feigelson, P. (1957). J. Biol. Chem. 226, 1001.

Marshall, A.M., Georges, R.J., Clarke, B.F., Whitby, L.G., Duncan, L.J.P. (1970). Metabolism. 19, 114.

Masturzo, M., and Negro, L. (1953). Biol. Sper. 29, 256.

McCance, R.A. (1926). Biochem. J. 20, 1111.

McCance, R.A., and Lawrence, R.D. (1929). Med. Res. Coun, Spec. Rep. Ser. No. 135.

Menzies, I.S., and Seakins, J.W.T. (1969). In Chromatographic and Electrophoretic Techniques, edited by Smith, I. Vol. I. 3rd Edition. William Heinemann. Medical Books Ltd.

Moczar, E., Moczar, M., Schillinger, G., Robert, L. (1967). J. Chromatogr. 31, 561.

Montreuil, J., and Boulanger, P. (1953). C.R. Acad. Sci. Paris. 236, 337.

Montreuil, J. (1960). Bull. Soc. Chim. Biol. 42, 1399.

Morgan, W.J.T. (1962). Ann. N.Y. Acad. Sci. 106, 177.

Nemec, J., Kefurt, K., and Jary, J. (1967). J. Chromatogr. 26, 116.

Ninnis, L., and Ninnis, M.B. (1957). Prakt. Akad. Atherion. 32, 414. Chem. Abstr. (1960). 54, 7736.

Oates, M.D.G. and Schrager, J. (1967). J. Chromatogr. 28, 232.

Oguri, K., Ida, S., Yoshimura, H., and Tsukamoto, H. (1970). Chem. Pharm. Bull. 18, 2414.

Orr, W.F., and Minot, A.S. (1952). Arch. Neurol & Psychiat. 67, 483.

Ovodov, YU.S., Evutshenko, E.V., Vaskovsky, V.E., Ovodova, R.G. and Solov'eva, T.F. (1967). J. Chromatogr. 26, 111.

Owen, O.E., Felig, P., Morgan, A.P., Wahren, J., and Cahill, G.F. (1969). J. Clin. Invest. 48, 574.

Partridge, S.M. (1948). Biochem. J. 42, 238.

Pallavicini, J.C., Gabriel, O., di Sant Agnese, P.A., and Buskirk, E.R. (1962). Ann. N.Y. Acad. Sci 106, 330.

Pigman, W., and Tettamanti (1968). In Biochemistry of glycoproteins and related substances, Part II. p. 117. edited by Rossi, E., and Stoll Basel, E., Karger, S. New York.

Pitkanen, E., Pitkanen A. and Perheentupa. J. (1964). Ann. Med. exp. Fenn. 42, 65.

Pittera, A., Cassia, B., and Fertilo, S. (1963). Arch. Studio. Fisiopatol. Clin. Ricambio. 27, 85. Chem. Abstr. (1964). 60, 4441 h.

Preece, I.A. and Hobkirk, R. (1953). J. Inst. Brew. 59, 385.

Pridham, J.B. (1956). Anal. Chem. 28, 1967 .

Reid, P.E., Donaldson, B., Secret, D.W. and Bradford, B. (1970). J. Chromatogr. 47, 199.

Richey, J.M., Richey, H.G., Jr. and Schraer, R. (1964). Anal. Biochem. 9, 272.

Roden, L. (1968). In Biochemistry of glycoproteins and related substances edited by, Rossi, E. and Stoll, E. Cystic Fibrosis part II, p 185. Basel-S. Karger - New York.

Roden, L. (1969). In pentoses and pentols edited by Horecker, B.L., Lang, K., and Takagi, Y. Springer - Verlag. Berlin. Heidelberg. New York.

Roden, L., and Lindahl, U. (1965). Federation Proc. 24, 606.

Ronzoni, E., Walk, S.m., Lam, R.L., and Gildea, E.F. (1955). Neurology 5, 412.

Rosevear, J.W., and Smith, E.L. (1961). J. Biol. Chem. 236, 425.

Rubin, M. (1970). In Advances in Clinical Chemistry 13, 211.

Salkowski, E., and Jastrowitz, M. (1892). Zentralbl. Med. Wissenesch. 30, 337.

Sarker, K.P. (1963). Sci. Cult. 29, 51. Chem. Abstr. (1963). 59, 5498 C.

Sawardeker, J.S., Sloneker, J.H. and Jeanes, A. (1965). Anal. Chem. 37, 1602.

Schachter, H., Sarney. J., McGuire, E.J. Roseman, S., (1969). J. Biol. Chem. 246, 4785.

Schersten, B. and Tibling, G. (1967). Clin. Chim. Acta. 18, 383.

Schiwara, H., Domschke, W., and Domagk, G.I. (1968). Hoppe - Seyer's. Z. 349, 1575.

Schrager, J. and Oates, M.D.G. (1968). In Biochemistry of glycoproteins and related substances, edited by Rossi, E., and Stoll, E. Cystic Fibrosis part II, p 270. Basel-S. Karger - New York.

Schweiger, A. (1962). J. Chromatogr. 9, 374.

Segal, S., and Foley, J. (1958). J. Clin. Invest. 37, 719.

Segal, S., and Topper, Y.J. (1957). Biochem. Biophys. Acta. 25, 419.

Segal, S. and Topper, Y.J. (1960). Biochem. Biophys. Acta. 42, 147.

Segal, S. Wyngaarden, J.B., and Foley, J. (1957). J. Clin. Invest. 36, 1383.

Selliere, G. (1908). C.r. Soc. biol. 64, 941.

Selliere, G. (1909). C.r. Soc. biol. 66, 691.

Shanon, J.A. (1938). Amer. J. Physiol. 122, 775.

Sharma, N.C., and Sur. B.K. (1969). Clin. Sci. 36, 317.

Sharon, N. (1966). Annual Review of Biochemistry. 35, 485.

Shellard, E.J. and Jolliffee, G.H. (1966). J. Chromatogr. 24, 76.

Shen, L., Grollman, E.F., and Ginsburg, V. (1968). Proc. Nat. Acad. Sci. 59, 224.

Smith, H.W. (1951). The Kidney. p. 101. New York. Oxford University Press.

Smith, M.G. (1962). Biochem. J. 83, 135.

Somogyi, M. (1927). J. Biol. Chem. 75, 33.

Southgate, D.A.T. (1965). Ph. D. Thesis, London University.

Southgate, D.A.T. and Durnin, J.V.G.A. (1970). Brit. J. Nutr. 24, 517.

Spiro, R.G. (1965). J. Biol. Chem. 240, 1603.

Spiro, R.G. (1970). Annual Review of Biochemistry. 39, 599.

Stefanis, De. V.A. and Ponte, Jr., J.G. (1968). J. Chromatogr. 34, 116.

Steinberg, T., Ortman, P., Poucher, R., Cochran, B., and Gwinup, G. (1967). Metabolism. 16, 40.

Stern, E.L. (1968). Federation Proc. 27, 596.

Stjernholm, R.L., and Noble, E.P. (1961). J. Biol. Chem. 236, 614.

Stjernholm, R.L., and Noble, E.P. (1963). Arch. Biochem. Biophys. 100, 200.

Stjernholm, R.L., Noble, E.P., Dimitrov, N.V., Morton, D.J., and Falor, W.H. (1969). J. Reticuloendothelial Soc. 6, 590.

Strecker, H.J., and Korkes, S. (1952). J. Biol. Chem. 196, 769.

Swartz, M.D. (1910-11). Trans. Connecticut Acad. Arts. Sci. 16, 274.

Sweeley, C.C., Bentley, R. Makita, M. and Wells, W.W. (1963). J. Am. Chem. Soc. 85, 2497.

Talukder, M.Q.-K. (1971). J. Chromatogr. 57, 391.

Tarr, H.L.A. (1955). Biochem. J. 59, 386.

Tominaga, F., Oka, K., and Yoshida, N. (1965). J. Biochem. 57, 717.

Tomoda, M., and Murayama, K. (1965). Japan J. Exptl. Med. 35, 159.

Touster, O. (1959). Amer. J. Med. 26, 724.

Touster, O., and Harwell, S.O. (1958). J. Biol. Chem. 230, 1031.

Touster, O., Hecht, S.O., Todd, W.M. (1960). J. Biol. Chem. 235, 951.

Touster, O., Reynolds, V.H., and Hutchison, R.M. (1956). J. Biol. Chem. 221, 697.

Tower, D.B., Peters, E.L., and Pogorelskin, M.A. (1956). Neurology. 6, 37 and 125.

Towne, J.C. and Spikner, J.E. (1963). Anal Chem. 35, 211.

Tunman, P., and Silberzahnm H. (1961). Z. Lebenson-Untersuch. U.-Forsch 115, 121. Chem. Abstr. (1961). 55, 22645 h.

Vidershayn, G.Y. and Rosenfeld, E.L. (1969a). Vop. Med. Khim. 15, 203. Chem. Abstr. (1969). 71, 11344 g.

Vidershayn, G.Y., and Rosenfeld, E.L. (1969b). Biokh. 34, 348.

Vitek, V., and Vitek, K. (1970). Biochemical Medicine 4, 282.

Vomhof, D.W. and Tucker, T.C. (1965). J. Chromatogr. 17, 300.

Walborg, E.F. and Lantz, R.S. (1968). Anal. Biochem. 22, 123.

Wardi, A.H., Allen, W.S., Turner, D.L., and Stary, Z. (1966). Arch. Biochem. Biophys. 117, 44.

Walton, J.N., and Latner, A.L. (1954). Arch. Neurol & Psychiat. 72, 362.

Watkins, W. (1966). Science. 152, 172.

Weicker, H., and Grasslin, D. (1966). Nature. 212, 715.

Wells, W.W. (1970). Methods in Medical Research 12, 115.

Wells, W.W., Chin. T., and Weber, B. (1964). Clin. Chim. Acta. 10, 352.

Wessler, E. (1971). Biochem. J. 122, 373.

Wheeler, H.J., and Tollens, B. (1889). Ann. 254, 329.

Whistler, R.L., and Durso, D.F. (1950). J. Am. Chem. Soc. 72, 677.

White, A.A., and Hess, W.C. (1956). Arch. Biochem. Biophys. 64, 57.

Wilkinson, J.F. (1957). Nature. 180, 995.

Winzler, R.J. (1958). In Ciba foundation symposium on the chemistry and biology of mucopolysaccharides, edited by Wolstenholme, G.E.W. and O'Connor, M. Churchill. London.

Wolfrom, M.l., De Lederkremer Rosa, M., and Schwab, G. (1966). J. Chromatogr. 22, 474.

Wolfrom, M.L., Patin, D.L., De Lederkremer Rosa, M. (1965). J. Chromatogr. 17, 488.

Wood, L.A. (1954). J. Pharmacology. 112, 158.

Wood, W.A., McDonough, M.J. and Jacobs, L.B. (1961). J. Biol. Chem. 236, 2190.

Wyngaarden, J.B., Segal, S., and Foley, J.B. (1957). J. Clin. Invest. 36, 1395.

Young, D.S. (1970). Amer. J. Clin. Path. 53, 803.

# APPENDIX

I. Systems additional to those described in the main text which will effectively separate fucose from other urinary sugars.

II. Unsuccessful thin-layer chromatographic systems.

III. Experiments on the recovery of urinary monosaccharides especially aldopentoses using the charcoal desalting procedure of Hughes & Whelan.

IV. Estimations of xylose and fucose in the same spot of thin-layer chromatogram by the dichromatic method of Bell.

V. Published papers.

# APPENDIX I

## SYSTEMS ADDITIONAL TO THOSE DESCRIBED IN THE MAIN TEXT WHICH WILL EFFECTIVELY SEPARATE FUCOSE FROM OTHER URINARY SUGARS

The following additional systems will separate fucose from all the sugars present in normal human urine. In each of the following, 20 x 20 cm plates and 0.5 mm layer thickness were used. Slurries were prepared in proportion of Kieselguhr G (1) and water (2.5).

A. On plates coated with kieselguhr G made up with 0.15 M- $NaH_2PO_4$

(1) Ethyl acetate-methanol-butan-1-ol-water (8: 2: 1: 0.5)

(2) Ethyl acetate-methanol-propan-2-ol-butan-1-ol-water (8: 1: 1: 1: 0.5)

(3) Ethyl acetate-methanol-butan-1-ol-water (16: 3: 3: 3)

(4) Butan-1-ol-methanol-acetic acid-water (5: 2.5: 1.5: 1)

(5) Butan-1-ol-acetic acid-water (8: 3: 2)

B. As in A. but using 0.20 M- $NaH_2PO_4$

Ethyl acetate-butan-1-ol-methanol-water (14: 4: 3: 1)

C. As in A. but using 0.30 M- $NaH_2PO_4$

(1) Butan-1-ol-propan-2-ol-water (8: 3: 1)

(2) Ethyl acetate-propan-2-ol-butan-1-ol-water (8: 2: 1: 1)

(3) Ethyl acetate-methanol-butan-1-ol-water (8: 2: 1: 0.5)

(4) Ethyl acetate-methanol-propan-2-ol-butan-1-ol-water (8: 1: 1: 1: 0.5)

APPENDIX II

UNSUCCESSFUL THIN-LAYER CHROMATOGRAPHIC SYSTEMS

This appendix records 72 thin-layer chromatographic systems examined which proved unsuccessful in separating arabinose, xylose and ribose (a) one from another and (b) from the other sugars detected in deionised urines from healthy fasting subjects. Many of these systems were examined or modified because of published results (see sections on review of paper chromatography and t.l.c.) which were said to separate one or two out of the three aldopentoses; when attempts were made to separate all three, one or more were found to coincide either with another aldopentose or with hexoses. Only pure sugars were run. $R_F$ values of the sugars were not recorded. Thin-layer plates, (20 x 20 cm) (adsorbent 0.5 mm thick) except mentioned otherwise were developed at room temperature (18 - 20°) by one dimensional ascending technique. Slurries were prepared in proportion of adsorbent (1) and water (2.5).

A. Cellulose (Chromedia) plates - with or without filter paper pads to assist longer running. Useless solvent systems were:

(1) Propan-2-ol-water (4: 1)

(2) Butan-1-ol-ethyl acetate-propan-2-ol-acetic acid-water (3.5: 10: 6: 3.5: 3)

(3) Chloroform-acetic acid-water (3: 3.5: 0.5)

(4) Chloroform-acetic acid-water (4.5: 3.5: 0.5)

(5) Chloroform-acetic acid-water (4.5: 6.5: 0.5)

(6) Pyridine-ethyl acetate-acetic acid-water (5: 5: 1: 3)

(7) Chloroform-acetone-acetic acid-water (4.5: 3: 6.5: 0.5)

(8) Ethyl acetate-propan-2-ol-water (5: 3: 1.5)

(9) Ethyl acetate-acetic acid-methanol-water (6: 1.5: 1.5: 1)

(10) Ethyl acetate-propan-2-ol-water (4: 1: 2)

(11) Butan-1-ol saturated with water.

(12) Butan-1-ol-acetone-acetic acid-water (4: 5: 0.5: 1)

(13) Propan-2-ol-butan-1-ol-water (7: 1: 2)

(14) Butan-1-ol saturated with water-propan-2-ol (9: 1)

(15) Butan-1-ol-acetic acid-water (4: 1: 5)

(16) "Amyl alcohol" saturated with water.

(17) "Amyl alcohol" saturated with water-propan-2-ol (3: 2)

(18) "Amyl alcohol" saturated with water-propan-2-ol-acetic acid (3: 1: 1)

(19) Propan-2-ol-propan-2-yl acetate-water (2: 4: 1)

(20) Toluene-butan-1-ol saturated with water (1: 2)

B. Cellulose made up with boric acid (0.21 gm/100 ml water)

Useless solvents were:

(1) Ethyl acetate-pyridine-water (4: 3: 2)

(2) Ethyl acetate-acetic acid-methanol-water (12: 3: 3: 2)

C. Silica (Kieselgel H, Merck) made up with boric acid (0.21 gm 100 ml water)

Useless solvents were:

(1) Ethyl acetate-pyridine-water (4: 3: 2)

(2) Ethyl acetate-acetic acid-methanol-water (6: 3: 3: 2)

(3) Propan-2-ol-water (4: 1)

D. Silica (Kieselgel H) made up with 0.3 M-$Na_2H\ PO_4$

Useless solvents were:

(1) Butan-1-ol-acetone-water (4: 5: 1)

(2) Ethyl acetate-propan-2-ol-water (4: 1: 2)

(3) Ethyl acetate-acetic acid-water (6: 3: 2)

(4) Butan-1-ol-ethanol-water (2: 1: 1)

(5) Butanol-methanol-water (1: 3: 1)

(6) Ethyl acetate-propan-2-ol-acetic acid-water (4: 1: 0.5: 2)

(7) Butan-1-ol-methanol-water (5: 3: 1)

E. Kieselguhr G (Merck)

Useless solvents were:

(1) "Amyl alcohol" saturated with water.

(2) "Amyl alcohol" saturated with water-propan-2-ol (3: 2)

(3) "Amyl alcohol" saturated with water-propan-2-ol-acetic acid (3: 1: 1)

F. Kieselguhr G made up with 0.02 M sodium acetate

Useless solvents were:

(1) Propan-1-ol-propan-2-yl acetate-water (2: 4: 1)

(2) Propan-2-ol-butan-1-ol-water (7: 1: 2)

(3) A double run using (a) Propan-2-yl acetate saturated with water and (b) Propan-2-ol-ethyl acetate-water (1: 4: 0.5)

(4) Ethyl acetate-propan-2-ol-water (9: 1: 1)

(5) Ethyl acetate saturated with water.

(6) Ethyl acetate-propan-2-ol-methanol-water (9: 1: 1: 1)

(7) Two dimensional (a) butan-1-ol-methanol-water (4: 5: 1)

(b) Ethyl acetate-propan-2-ol-water (4: 1: 0.5)

(8) Ethyl-methyl ketone-ethyl acetate-propan-2-ol-water (2: 2.5: 1: 0.5)

(9) Ethyl methyl ketone saturated with water.

(10) Water saturated with boric acid.

G. Kieselguhr G made up with 0.1 M sodium acetate

Ethyl acetate-propan-2-ol-water (4: 1: 0.5) proved useless.

H. Kieselguhr G made up with 0.02 M sodium borate

Solvents proving useless were:

(1) Ethyl acetate-propan-2-ol-water (4: 1: 0.5)

(2) Butan-1-ol-methanol-water (4: 2.5: 0.5)

I. Kieselgel H made up with $Na_2H\,PO_4$

Useless solvents were:

(1) Butan-1-ol-acetone-water (4: 5: 1)

(2) Ethyl acetate-propan-2-ol-water (4: 1: 2)

(3) Ethyl acetate-acetic acid-water (6: 3: 2)

(4) Butanol-methanol-water (2: 1: 1)

(5) Butanol-methanol-water (5: 3: 1)

(6) Ethyl acetate-propan-2-ol-acetic acid-water (4: 1: 0.5: 2)

J. Kieselguhr G made up with 0.1 M or 0.2 M $Na_2H PO_4$

Useless Solvents were:

(1) Ethyl acetate-propan-2-ol-water (4: 1: 0.5)

(2) Ethyl acetate-methanol-butan-1-ol-water (8: 2.1: 0.5)

K. Kieselguhr G made up with 0.3 M $Na H_2 PO_4$

Useless solvents were:

(1) Butan-1-ol-methanol-water (4: 2.5: 0.5)

(2) Ethyl acetate-methanol-butan-1-cl (4: 1: 0.5)

L. Cellulose (Chromedia) (1 part) + Kieselguhr G (2 parts) w/w made up with 0.15 M $Na H_2PO_4$

Useless solvents were:

(1) Ethyl acetate-propan-2-ol-water (4: 1: 0.5)

(2) Ethyl acetate-methanol-butan-1-ol-water (16: 3: 3: 3)

M. Kieselguhr G (2 parts) + Kieselgel G made up with 0.15 M $NaH_2PO_4$

Useless solvents were:

(1) Ethyl acetate-propan-2-ol-water (4: 1: 0.5)

(2) Ethyl acetate-methanol-butan-1-ol-water (16: 3: 3: 3)

(3) Butan-1-ol-methanol-water (8: 5: 1)

(4) Butan-1-ol-methanol-acetic acid-water (15: 7.5: 4.5: 3)

(5) Butan-1-ol-acetic acid-water (8: 3: 2)

N. Kieselguhr G (2 parts) + Alumina G (1 part) made up with 0.15 M $NaH_2PO_4$

Useless solvents were:

(1) Butan-1-ol-formic acid-water (9: 2: 2)

(2) Butan-1-ol-propan-2-ol-acetic acid-water (7: 3: 3: 2)

O. Kieselguhr G (1 part) + Alumina G (1 part) made up with 0.3 M $NaH_2PO_4$

Useless solvents were:

(1) Ethyl acetate-propan-2-ol-water (4: 1: 0.5)

(2) Ethyl acetate-methanol-propan-2-ol-butan-1-ol-water (8: 1: 1: 1: 0.5)

(3) Butan-1-ol-methanol-water (8: 5: 1)

(4) Butan-1-ol-acetic acid-water (8: 3: 2)

P. "plaster of Paris"

Useless solvent proved to be Ethyl acetate-propan-2-ol-water (4: 1: 0.5)

## APPENDIX III

## EXPERIMENTS ON THE RECOVERY OF URINARY MONOSACCHARIDES ESPECIALLY ALDOPENTOSES USING THE CHARCOAL DESALTING PROCEDURE OF HUGHES & WHELAN (1958).

The authors quoted above found that milligram amounts of several monosaccharides could be recovered at proportions between 94 to 101% from salt-containing solutions by passing these through a column packed with celite and a special charcoal (Ultrasorb S.C. 20/240 obtained from British Carbo-Norite Union Ltd. of Grays, Essex). From these columns, after washing out salts with a specified volume of water, the adsorbed monosaccharides were eluted by 0.5% ethanolic water.

As this procedure appeared to offer a convenient method of desalting urine for subsequent t.l.c. I examined it using two separate batches of carbo-norite. The analytical method employed on the appropriate eluate was that of Dische & Borenfreund (1956).

Initial method of desalting using the first batch of Carbo-norite

Into columns (2 cm in diameter) a mixture of the charcoal (5 g) and celite (3 g) was packed wet. From such columns the eluate-rate was very close to 0.25 ml/min. To wash out salts, 100 ml of water was passed through the column; this was followed by 100 ml of 5% ethanolic water to elute the monosaccharides.

Experiments with a "synthetic urine":

An imitation of one litre of urine was made up containing urea (15 g), uric acid (1 g), citric acid (0.3 g), oxalic acid (0.02 g), $KH_2PO_4$ (3.0 g), NaCl (12 g), $CaCl_2$ (0.5 g), $Na_2SO_4$ (2 g), arabinose and xylose (each 25 mg), ribose (30 mg), fucose (25 mg), glucose and fructose and galactose (each 100 mg). The total aldopentose concentration was therefore 80 µg/ml. Five ml samples of the synthetic urine were introduced into three separate columns each of which was "washed" with 100 ml of water. The sugars were then desorbed by passing through 100 ml of 5% ethanolic water. The concentrations of the total pentoses in the ethanolic eluates were measured as stated above and the Table 1 shows my experimental results:-

TABLE 1

Recovery of aldopentoses using charcoal column

| Column No. | Total aldopentose before column treatment found, µg/ml | Total aldopentose after column treatment found, µg/ml | Recovery % |
|---|---|---|---|
| 1 | 78 | 48 | 62 |
| 2 | 78 | 44 | 86 |
| 3 | 78 | 46 | 59 |

As the above low recovery was considered to be possibly due, at this microgram level, to the amount of water used in washing out the salts

from the column, an experiment was made to examine this suggestion, as follows:- (a) Total aldopentose was measured on the solution introduced into the column, (b) the same measurement was made on the "salt-removing" eluate of 100 ml of water, and (c) the same measurement was made after eluting the water-washed column by 5% aqueous ethanol. Results are shown in Table 2.

TABLE 2

Loss of aldopentoses in water washing and overall recovery

| Samples of "Artificial urine" | Total aldopentose (µg/ml) | | | Overall Recovery % |
|---|---|---|---|---|
| | Before column treatment | In washings by 100 ml of water (% loss in brackets) | In eluate (5% EtOH) | |
| 1 | 76 | 20 (26%) | 43 | 59 |
| 2 | 76 | 19 (25%) | 46 | 61 |

These recoveries, although low, are consistent and, at the ug level washing out of salts results in 25% loss. Hughes & Whelan (1958) reported that prolonged washing with water, of their columns would slowly begin to elute monosaccharides (within mg level).

Recoveries using two concentrations of aqueous methanol were examined and are described in Table 3. These measurements of total aldopentoses (eluted from carbonorite columns) were done without previous aqueous elution of salts.

TABLE 3

Micrograms of total aldopentose recovered from synthetic urine starting with 78 µg using two concentrations of aqueous methanol

| Ethanolic solution | µg/ml and % of aldopentose recovered after elution by aqueous MeOH of % stated | | | |
|---|---|---|---|---|
| | 2.5% | 5% | 2.5% | 5% |
| 1st 100 ml | 60 | 65 | 77 | 83 |
| 2nd 50 ml | 10 | 6 | 12 | 7 |
| 3rd 50 ml | 4 | 3 | 5 | 5 |
| Total recovery | 74 µg | 74 µg | 94% | 95% |

Thus, with this batch of charcoal, no difference in the recovery of the total aldopentose was found on elution by 2.5% and 5% aqueous methanol provided a sufficient elution volume was used.

The Second batch of Carbonorite

Three chromatographic columns were arranged as for the first batch of carbonorite. The synthetic urine was analysed to contain 77 µg/ml of total aldopentose. The experiment is outlined in Table 4.

TABLE 4

Total aldopentose found after treatments given below

| Treatments | Column 1 | Column 2 | Column 3 |
|---|---|---|---|
| In eluate of 100 ml of water | 0 | 8 | 2 |
| In eluate of 125 ml of 2.5% aq. MeOH | 58 | 52 | 53 |
| % Recovery of total aldopentose | 75 | 67 | 69 |

These recoveries, although low are reasonably consistent.

Elution of aldopentoses by 30% aqueous methanol:

On the synthetic urine, treated as above with water to remove salts, subsequent elution of the columns by 30% aqueous methanol (50 ml/column) yielded an eluate containing 91% of the total aldopentose originally introduced. It was therefore decided to repeat this treatment on 2 specimens of human urine on which the total aldopentose could be measured (Bell et al. 1972). The results are shown in Table 5.

TABLE 5

Recoveries of urinary aldopentose

| | Total aldopentose, µg/ml | |
|---|---|---|
| | Urine 1 | Urine 2 |
| Before treatment | 48 | 43 |
| Found, in 100 ml of water washings | 23 | 26 |
| Found, in 75 ml of 30% MeOH eluate | 32 | 19 |
| % Recoveries in methanolic eluate | 66 | 44 |

These recoveries, in the alcoholic eluate, are poor and irregular considering that the same batch of carbo-norite and the same solvents were used.

Investigation of the recoveries of total aldopentoses after deionisation of urine by ion exchange columns of IR 120 ($H^+$) and IR 400 (acetate)

As stated in Chapter 3 it was eventually found possible to deionise urine with insignificant loss of sugars. The following Table 6 shows my original findings when total aldopentose was measured on six aliquots from the same urine.

TABLE 6

Recovery of aldopentose using ion-exchange resin column

| Resin columns | Total aldopentose, μg/ml | | % Recovery |
|---|---|---|---|
| | Before deionisation | After deionisation | |
| 1 | 28 | 25 | 89 |
| 2 | 37 | 35 | 96 |
| 3 | 37 | 35 | 96 |
| 4 | 37 | 37 | 100 |
| 5 | 34 | 29 | 85 |
| 6 | 32 | 30 | 94 |
| | | Mean recovery | 93 |

It was therefore decided that deionisation of urine, to give satisfactory results at the microgram level, must be effected by suitable ion-exchange resin.

## APPENDIX IV

## ESTIMATION OF XYLOSE AND FUCOSE IN THE SAME SPOT OF THIN-LAYER CHROMATOGRAMS BY THE DICHROMATIC METHOD OF BELL (1966)

Thin-layer chromatography on acetate plates (this thesis) separates arabinose and ribose from other monosaccharides but not xylose from fucose, an effect also seen in many other t.l.c. systems. (see Appendix II). Measurements of fucose and xylose in the same spot from the t.l. acetate plate were attempted on the basis that these sugars yield different colours (pink for xylose and yellow for fucose) with the PABA reagent (Bell 1966). Pentoses develop an intense pink colour on standing in the dark for a few hours ( $\lambda$ max at 540 nm. Then has a smaller peak at 370 nm). Aldohexoses and fucose gave intense $\lambda$ max at 370 nm and a small absorption at 540 nm. Similar absorption peaks were obtained for xylose and fucose with PABA spraying reagent after extracting the sugars from thin-layer acetate plates. Xylose showed a double peak of maximum absorption (fig. 18) at 520 nm and again at 370 nm. Fucose gave intense $\lambda$ max at 370 nm. By measuring extinctions at 370 nm and at 520 nm, it was possible to measure fucose and xylose in mixtures by comparison with standard solutions using the expression devised by Bell (1966):

$$\text{Xylose} = \frac{a_1 y - a_2 x}{a_1 b_2 - a_2 b_1} \times \text{known amount of xylose used as standard.}$$

$$\text{Fucose} = \frac{b_2 x - b_1 y}{a_1 b_2 - a_2 b_1} \times \text{known amount of fucose used as standard.}$$

Where -

$x$ = Extinction of mixture at 370 nm

$y$ = Extinction of mixture at 520 nm

$a_1$ = slope of standard curve for hexose at 370 nm

$b_1$ = slope of standard curve for pentose at 370 nm

$a_2$ = slope of standard curve for hexose at 520 nm

$b_2$ = slope of standard curve for pentose at 520 nm

Following are the results of experiments with known amounts of xylose and fucose which were applied to the acetate plate at the same spot. No development of the t.l.c. plates was done in solvent systems. Colours were developed on the plate after spraying with PABA reagent. Extraction of the coloured spots followed as described in Chapter 3 of this thesis.

TABLE: Percentage recovery of xylose and fucose from mixtures of known composition

| Experiments | Known sugar mixtures ug | Amount recovered ug | % recovery |
|---|---|---|---|
| 1. | Xylose 20 | 12 | 60 |
| | Fucose " | 26 | 130 |
| 2. | Xylose " | 19 | 95 |
| | Fucose " | 19 | 95 |
| 3. | Xylose " | 22 | 110 |
| | Fucose " | 19 | 95 |
| 4. | Xylose 20 | 18 | 90 |
| | Fucose 10 | 11 | 110 |

| Experiments | Known sugar mixtures | | Amount recovered ug | % recovery |
|---|---|---|---|---|
| 5. | Xylose | 20 | 22 | 110 |
| | Fucose | 5 | 3 | 60 |
| 6. | Xylose | 20 | 14 | 93 |
| | Fucose | 5 | 2 | 40 |
| 7. | Xylose | 15 | 9 | 60 |
| | Fucose | " | 19 | 126 |
| 8. | Xylose | " | 17 | 113 |
| | Fucose | " | 12 | 80 |
| 9. | Xylose | " | 9 | 60 |
| | Fucose | " | 22 | 146 |
| 10. | Xylose | " | 21 | 140 |
| | Fucose | " | 12 | 80 |
| 11. | Xylose | 15 | 19 | 126 |
| | Fucose | 15 | 15 | 100 |
| 12. | Xylose | " | 16 | 106 |
| | Fucose | " | 13 | 86 |
| 13. | Xylose | 15 | 15 | 100 |
| | Fucose | " | 15 | 100 |
| 14. | Xylose | " | 22 | 147 |
| | Fucose | " | 8 | 53 |
| 15. | Xylose | " | 18 | 120 |
| | Fucose | " | 22 | 80 |
| 16. | Xylose | " | 20 | 132 |
| | Fucose | " | 11 | 73 |
| 17. | Xylose | " | 18 | 120 |
| | Fucose | " | 13 | 86 |
| 18. | Xylose | " | 14 | 93 |
| | Fucose | " | 10 | 66 |

| Experiments | Known sugar mixtures ug | Amount recovered ug | % recovery |
|---|---|---|---|
| 19. | Xylose " | 16 | 106 |
| | Fucose " | 14 | 93 |
| 20. | Xylose 15 | 10 | 66 |
| | Fucose 10 | 13 | 130 |
| 21. | Xylose 15 | 12 | 80 |
| | Fucose 10 | 14 | 140 |
| 22. | Xylose 15 | 14 | 93 |
| | Fucose 10 | 12 | 120 |
| 23. | Xylose 15 | 14 | 93 |
| | Fucose 5 | 2 | 40 |
| 24. | Xylose 10 | 8 | 80 |
| | Fucose 15 | 21 | 140 |
| 25. | Xylose 10 | 9 | 90 |
| | Fucose 10 | 12 | 120 |
| 26. | Xylose 10 | 8 | 80 |
| | Fucose " | 7 | 70 |
| 27. | Xylose " | 8 | 80 |
| | Fucose " | 6 | 60 |
| 28. | Xylose " | 9 | 90 |
| | Fucose " | 12 | 120 |
| 29. | Xylose 5 | 4 | 80 |
| | Fucose 10 | 12 | 120 |
| 30. | Xylose 5 | 4 | 80 |
| | Fucose 5 | 5 | 100 |
| 31. | Xylose " | 4 | 80 |
| | Fucose " | 5 | 100 |
| 32. | Xylose " | 5 | 100 |
| | Fucose " | 6 | 120 |

In these experiments xylose showed average recovery of 93% (range 60 - 146%) and fucose 97% (range 40 - 146%). This method was not however finally applied to measure fucose and xylose in urine. The recovery range would have been considerably worse when full-treatment of the sugars would be given as for urine samples. However t.l.c. quantitative separation of fucose from xylose was eventually achieved.

# APPENDIX V

# PUBLISHED PAPERS

Reprinted from

Journal of Chromatography
Elsevier Publishing Company, Amsterdam - Printed in The Netherlands
CHROM. 4703

# THIN-LAYER QUANTITATIVE CHROMATOGRAPHY OF ARABINOSE, RIBOSE AND XYLOSE IN THE PRESENCE OF OTHER SUGARS

D. J. BELL AND M. Q.-K. TALUKDER
*Physiology Department, University Medical School, Edinburgh EH8 9AG (Great Britain)*

(Received February 24th, 1970)

SUMMARY

We describe a rapid and unequivocally distinct separation of arabinose, ribose and xylose from all sugars, except fucose, which are normally found in biological fluids. The method can be applied quantitatively, with reasonable accuracy, to amounts of up to 80 $\mu$g of each aldopentose.

## INTRODUCTION

Having found that arabinose, ribose and xylose are invariably present in the urine of fasting normal human subjects we sought for a simple and rapid way to measure the amounts of these sugars. We considered that the development of a procedure which could be applied in a clinical laboratory might prove of value in studying diseases involving connective tissue since it is now well-established that D-xylose and L-arabinose occur as components of glycoproteins associated with this tissue.

The three aldopentoses are not readily separated on paper, if at all, by the published procedures[1-3] and many hours of development, with concomitant spread of the spots, are required, so that quantitative analyses become difficult. Thin layers of Silica Gel G do not allow good separation between arabinose, xylose and mannose[4-6] and analogous overlapping takes place with crystalline cellulose[7].

This paper describes a slight modification of the thin-layer system of STAHL AND KALTENBACH[8] using Kieselguhr G made up with 0.03 $M$ sodium acetate; two developments of 55 min each with suitable proportions of ethyl acetate, propan-2-ol and water give wide spatial separation of the three aldopentoses from one another and from all the other sugars found in normal urine with the exception of fucose. The latter, as far as we can see, occurs only in minor amounts in normal fasting urine but its presence can be detected by a qualitative differential spray reagent. We have not been able to separate xylose from fucose. The clear-cut separation of the aldopentose spots enables quantitative measurements to be made.

Using the unpleasant mixture of ethyl acetate–pyridine–water, it is possible on

cellulose to show separations which are qualitatively satisfactory but the resolution is not sufficient for quantitative purposes.

Our procedure has now been successfully used by colleagues in other laboratories.

## EXPERIMENTAL AND RESULTS

### *Solvents*

Alcohols were distilled over potassium hydroxide and silver oxide. Ethyl acetate was distilled after keeping over anhydrous potassium carbonate. Glacial acetic acid was distilled over potassium permanganate.

### *Sugars*

Commercial samples were recrystallised, D-xylose and D-ribose from propan-2-ol and L-arabinose from aqueous propan-2-ol. Stock solutions (5 mg/ml) were prepared in water saturated with benzoic acid.

### *Reagents*

The reagents were commercial samples and usually did not require further purification. 4-Methoxyaniline (*p*-anisidine) hydrochloride was prepared in the laboratory.

### *Chromatographic plates*

Kieselguhr G (Merck) (one part) blended for 1 min with 0.03 *M* sodium acetate (three parts) was spread at a thickness of 0.5 mm. The plates were allowed to dry at room temperature for 24 h and were stored at the humidity and temperature of the laboratory.

### *Application of sugar solutions*

Streaks, about 1.5 cm long, were applied uniformly from a 5-$\mu$l graduated pipette. Amounts of 1, 2 and 3 $\mu$l were typically used. Water was dried off in a current of warm air.

### *Development*

The tanks were lined with filter paper as a routine but no previous vapour saturation was found to be necessary. The developing solvent, slightly modified in proportions from that of STAHL AND KALTENBACH[8] consisted in ethyl acetate–propan-2-ol–water (4:1:0.5). At room temperature the solvent was allowed to run the full length of the plate (20 cm in 55 min). If fructose was present, or if quantitative results were required, the solvent was evaporated in a current of air (15 min) and a second development was done to separate arabinose from the hexulose.

The developing solvent lasts for 48 h.

### *Spray reagents and revelation of sugars*

Application was made using the Shandon Laboratory Spray Gun.

*Attempts to reveal the sugar spots.* Sprays commonly used on paper, cellulose powder or silica, when applied to the buffered kieselguhr, despite attempts to over-

some the buffer by increasing the acidity of the reagents, etc., generally proved quite unsatisfactory. Reagents containing aniline salts or salts of 4-aminobenzoic acid[9] gave very faint colours. Naphthoresorcinol[4–6] gave generally blue colours with a poor background. A range of indicators of suitable p$K$ values with boric acid failed completely to show up any sugars. Acid phloroglucinol likewise proved unsatisfactory. However, a new reagent proved to give excellent qualitatively differential results but it was impossible to extract the colours without immediate fading; however, a fairly permanent staining took place with PRIDHAM's[10] *p*-anisidine spray (see below).

*New qualitative spray.* Stannous chloride added to the reagent used by BELL[9] for assay of hexoses and pentoses in aqueous solution gave intense colours as follows: pentoses, cerise; aldohexoses, brown-yellow; hexuloses, yellow; 6-deoxyaldohexoses, yellow or yellow-pink. The reagent consisted in 4-aminobenzoic acid (2 g), 3-carboxy-4-hydroxybenzenesulphonic acid (3 g) and $SnCl_2$ (1 g) in 100 ml of 80% aqueous acetic acid. The solution was filtered before use. Air-dried plates were sprayed and, without further drying, were heated for 15 min at 100°.

*Quantitative spray.* PRIDHAM's[10] reagent, devised for paper work, gave a stable colour which could be extracted from the kieselguhr. It consisted in 4-methoxyaniline (*p*-anisidine) hydrochloride (1 g) dissolved in MeOH (5 ml) containing sodium dithionite (100 mg) and butan-1-ol (95 ml). The air-dried plate was sprayed in four directions, allowed to dry in a current of air and then heated at 130° for 15 min when all the classes of sugar showed as brownish spots. (This spray gives selective colours when used on paper.)

*$R_F$ and $R_G$ values.* Typical values are given in Table I.

TABLE I

$R_F$ AND $R_G$ VALUES FOR EIGHT MONOSACCHARIDES

| *Sugar* | *Typical $R_F$ values × 100* | *Typical $R_G$ values × 100* |
|---|---|---|
| Rhamnose | 78 | 536 |
| Ribose | 66 | 460 |
| Fucose[a] | 47 | 324 |
| Xylose[a] | 45 | 312 |
| Arabinose | 32 | 224 |
| Fructose | 25 | 170 |
| Glucose | 14 | 100 |
| Galactose | 11 | 80 |

[a] Fucose and xylose do not separate completely.

*Quantitative measurements*

Because the sugar spots were so widely separated it was possible completely to remove the coloured areas of the kieselguhr from the glass surface *in toto*. Equal areas of the kieselguhr which contained the stain were carefully transferred to centrifuge tubes; at the same time equal areas which contained no sugars were treated likewise to serve as blanks. To each sample was added 4 ml of 95% aqueous methanol containing 1 g of $SnCl_2$ per 100 ml. The tubes were stoppered and vigorously shaken for 10 min and the solid then packed on the centrifuge. The extinctions of the supernatant

solutions were then measured at 395 nm (which is the $\lambda_{max}$), against the "blank" supernatant, on an SP500 spectrophotometer. The colour was stable for about 3 h and the three aldopentoses gave almost identical extinctions in the range of 5 to 80 $\mu$g. Typical results are given in Table II.

TABLE II

TYPICAL EXTINCTIONS FOR THREE ALDOPENTOSES STAINED BY PRIDHAM'S REAGENT

| *Amount* ($\mu$g) | *Extinctions* | | |
|---|---|---|---|
| | *Arabinose* | *Xylose* | *Ribose* |
| 5 | 0.045 | 0.045 | 0.040 |
| 10 | 0.12 | 0.12 | 0.09 |
| 20 | 0.24 | 0.25 | 0.23 |
| 40 | 0.58 | 0.58 | 0.60 |
| 80 | 1.15 | 1.15 | 1.16 |

ACKNOWLEDGEMENTS

The authors are indebted to Mr. A. E. PINK and Dr. C. DONALD (Research and Development Department, I.C.I. Ltd., Grangemouth) for gifts of chemicals and to the Agricultural Research Council (D.J.B.) and the Government of East Pakistan (M.Q.-K.T.) for financial support.

REFERENCES

1 D. J. BELL, in K. PAECH AND M. V. TRACY (Editors), *Modern Methods of Plant Analysis*, Vol. 2, Springer-Verlag, Berlin, 1955, p. 1.
2 L. HOUGH AND J. K. N. JONES, in R. L. WHISTLER AND M. L. WOLFROM (Editors), *Methods in Carbohydrate Chemistry*, Vol. 1, Academic Press, New York, 1962, p. 21.
3 I. S. MENZIES AND J. W. T. SEAKINS, in I. SMITH (Editor), *Chromatographic and Electrophoretic Techniques*, Vol. 1, 3rd ed., Heinemann, London, 1969.
4 G. W. HAY, B. A. LEWIS AND F. SMITH, *J. Chromatog.*, 11 (1963) 479.
5 M. LATO, B. BRUNELLI, G. CIUFFINI AND T. MEZZETTI, *J. Chromatog.*, 36 (1968) 191.
6 J. NĚMEC, K. KEFURT AND J. JARÝ, *J. Chromatog.*, 26 (1967) 116.
7 M. L. WOLFROM, D. L. PATIN AND R. M. DE LEDERKREMER, *J. Chromatog.*, 17 (1965) 488.
8 E. STAHL AND U. KALTENBACH, *J. Chromatog.*, 5 (1961) 351.
9 D. J. BELL, *J. Chem. Soc., C*, (1966) 1638.
10 J. B. PRIDHAM, *Anal. Chem.*, 28 (1956) 1967.

[*Reprinted from the Proceedings of the Biochemical Society*, 17–18 *December* 1970.
Biochem. J., 1971, Vol. 122, No. 1, 24 P.]

## Fucose in Urine of Fasting Human Subjects

By D. J. BELL and M. Q.-K. TALUKDER. (*Department of Physiology, University Medical School, Edinburgh EH8 9AG, U.K.*)

By quantitative t.l.c. (Bell & Talukder, 1970) we observed that urines from fasting (12–15h) human volunteers invariably contained, besides galactose, glucose and fructose, the additional free monosaccharides ribose, xylose and arabinose. In experiments using a differential stain to reveal the sugar spots we found that fucose occupied the same chromatographic position as xylose. We could find no evidence of any chromatographic system in which fucose did not coincide with at least one of the accompanying urinary sugars (cf. Partridge, 1948).

One of us (M.Q.-K.T.) has now devised a rapid (65min) t.l.c. system that clearly separates fucose ($R_F$ 0.72) from all other monosaccharides of 'fasting' urine ($R_F$ 0.55 to 0.10); the method can be used quantitatively over the range 5–80$\mu$g. The plates are coated with Kieselguhr G (Merck) buffered with 0.15M-$NaH_2PO_4$ and developed with a mixture of ethyl acetate, methanol, butan-1-ol and water (16:3:3:2, by vol.). Sugars are revealed by spraying with aq. 90% acetic acid containing 1g of 4-aminobenzoic acid, 0.6g of sulphosalicylic acid (Bell, 1966) and 2g of $SnCl_2$. After heating at 110°C for 20min hexoses show up yellow and pentoses pink, enabling separation to be confirmed. Areas containing the 6-deoxy sugars are scraped off, extracted with 90% acetic acid containing 2% (w/v) of $SnCl_2$ and the extinction read at 370nm, when a stoicheiometric relationship holds between $E$ 0.04 (5$\mu$g) and 0.6 (80$\mu$g) for each sugar.

Results from normal subjects and some pathological subjects will be presented.

We are indebted to the Medical Research Council (D.J.B.) and the Government of East Pakistan (M.Q.-K.T.) for financial support.

Bell, D. J. (1966). *J. chem. Soc.* p. 1638.
Bell, D. J. & Talukder, M. Q.-K. (1970). *J. Chromat.* **49**, 469.
Partridge, S. M. (1948). *Biochem. J.* **42**, 238.

PRINTED IN GREAT BRITAIN BY WILLIAM CLOWES AND SONS LIMITED, LONDON, COLCHESTER AND BECCLES

Reprinted from

Journal of Chromatography
Elsevier Publishing Company, Amsterdam - Printed in The Netherlands
CHROM. 5262

# A RAPID QUANTITATIVE THIN-LAYER CHROMATOGRAPHIC SEPARATION OF FUCOSE FROM OTHER NEUTRAL MONOSACCHARIDES

## AN APPLICATION TO SEPARATE OTHER SUGARS OF PHYSIOLOGICAL INTEREST

M. Q-K. TALUKDER

*Physiology Department, University Medical School, Edinburgh EH8 9AG (Great Britain)*

First received December 15th, 1970; revised manuscript received January 18th, 1971)

SUMMARY

Fucose can be completely separated in 65 min from seven other neutral monosaccharides of biological interest on thin layers of Kieselguhr G buffered with 0.15 *M* sodium dihydrogen orthophosphate. It can be determined within the range 5 to 80 $\mu$g. Using a plate 35 cm long, each of the eight individual sugars can be well separated, one from another, in 9 h. This procedure is limited to mixtures containing an upper level of 15 $\mu$g of xylose and ribose. Mixtures of fucose and xylose can be determined quantitatively by the method described.

## INTRODUCTION

BELL AND TALUKDER[1] have reported a quantitative thin-layer chromatographic (TLC) separation of arabinose, xylose and ribose from rhamnose, fructose, glucose and galactose; they found it impossible to separate fucose from xylose. Little information is available on the chromatographic separation of fucose (paper or thin layer); however, since BELL AND TALUKDER (unpublished) found that free fucose is invariably present in urine from fasting normal human subjects it became important to separate this sugar from all accompanying monosaccharides. As far as the author is aware, the only separation of fucose from xylose so far reported is the slow running paper/phenol–ammonia procedure of PARTRIDGE[2]. Here however rhamnose and ribose coincide and the close $R_F$ values of ribose and fucose may not permit quantitative measurements. A two-dimensional TLC procedure[3] which separates fucose from other monosaccharides obtained on hydrolysis of glycoproteins, does not deal with other aldopentoses which occur in association with connective tissue. LATO *et al.*[4], in an intensive study using silica gel impregnated by either sodium acetate, sodium dihydrogen phosphate or disodium hydrogen phosphate, show a few separations between xylose and fucose with respect to differences in their $R_F$ values. These values are small and in such instances the sugar spots are reported to be diffuse or tailing. Other sugars in urine would tend to coincide either with xylose or fucose or both. Moreover, the closeness or overlapping of the spots of the various sugars would militate against quantitative applications of these systems. In their illustration (Fig. 1 p. 416) the only run with both fucose and

xylose shows convergence of the fucose and ribose spots, although in this instance xylose and fucose are clearly separated.

Fucose, as tritiated fucitol, has been separated[5] from the corresponding alditols resulting from the reduction of ribose, mannose and galactose; again other monosaccharides of interest were not considered.

Having found[1] that plates coated with Kieselguhr G buffered with sodium acetate and suitably developed will effectively separate the eight monosaccharides of interest, with the exception of xylose and fucose, the observations of OVODOV *et al.*[6] were noted. These authors made the point that successful sugar separations (on buffered silica) depended on the nature and concentration of the impregnating salt. TALUKDER (unpublished) examined silica, cellulose and alumina, buffered by borate, sodium acetate, sodium dihydrogen phosphate and disodium hydrogen phosphate without satisfactorily separating fucose from xylose. Success was eventually attained using Kieselguhr G buffered with 0.15 *M* sodium dihydrogen phosphate.

## EXPERIMENTAL AND RESULTS

### *Solvents*

Alcohols (either reagent quality or AR) were distilled over sodium hydroxide and silver oxide. If methanol still contained volatile bases, it was re-distilled over potassium hydrogen sulphate. Ethyl acetate was distilled after storage over anhydrous potassium carbonate. Acetic acid was distilled from potassium permanganate.

### *Sugars*

Aldopentoses were purified as described by BELL AND TALUKDER[1]. Rhamnose was recrystallized from ethyl acetate at room temperature. Fucose (D or L) was recrystallised from ethanol at 2°; to ensure freedom from pentose it was spotted on a kieselguhr plate and stained differentially by the 4-aminobenzoic acid–sulphosalicylic acid mixture of BELL[7] with addition of 2 % $SnCl_2$. One commercial sample did indeed contain a pentose. Glucose, galactose and fructose were commercial products.

Stock solutions (5 mg/ml) were prepared in saturated benzoic acid.

### *Spray reagents*

4-Methoxy aniline (*p*-anisidine) hydrochloride was prepared in the laboratory. Other materials were commercial products.

### *Chromatographic plates*

Kieselguhr G (Merck) (one part) was blended for 90 sec with 0.15 *M* sodium dihydrogen orthophosphate (2.5 parts) and the slurry spread to a thickness of 0.5 mm. The plates were allowed to dry at room temperature (18–20°) for at least 24 h and stored in presence of air at a temperature not exceeding 20°. If the storage temperature exceeds 20° the plates become useless.

### *Application of the sugar solutions*

Solutions were applied, from a 5 $\mu$l graduated micropipette in streaks of not more than 1 cm in length to the plates; not more than a total of 3 $\mu$l was applied uniformly at one time. Spots were dried by a current of warm air (75–80°).

*Development*

A considerable variety of solvent mixtures were examined; only the following ave satisfactory results: (1) ethyl acetate–methanol–butan-1-ol–water (16:3:3:2); 2) ethyl acetate–methanol–propan-2-ol–butan-1-ol–water (8:1:1:1:1); (3) ethyl cetate–methanol–butan-1-ol–water (16:3:3:1). These solvent mixtures were un-ltable for use after 48 h.

As found previously[1] no initial vapour saturation was necessary when using paper-lined tanks. The solvent front was allowed to run the full length of the plate at room temperature (65 min for 20 cm long plates with solvent mixtures 1 or 2). The 5 cm long plates required $3^3/_4$ h with solvent mixture 3 for a single run. While a single development sufficed qualitatively to separate fucose from all the other sugars, to obtain sufficient spatial separation of fucose, as well as all the other individual monosaccharides from each other except ribose and xylose, two developments were necessary. Single development showed urinary sugars to run slightly behind the corresponding standards. But a second development overcame this. Moreover, the sugar spots became more compact and well shaped with a second or third development. After each run the plates were dried at room temperature for 15 min and then heated at 100° for 5 min to vaporise the butanol. When cold, the plates were ready either for subsequent development or for spraying.

*Visualisation of the sugar spots*

In preliminary investigations a differential spray was used, based on the BELL's reagent[7] modified by the addition of 2 g/100 ml of $SnCl_2$ to stabilize the red colour of the pentoses and the yellows developed with the different classes of hexoses. This reagent cannot yet be used for accurate quantitative work because of small irregularities in the amounts of colour developed in the pentose spots. 6-Deoxyhexose spots showed a distinct pink tinge in the yellow which was formed on phosphate-buffered plates.

As before[1], butanolic 4-methoxyaniline containing sodium dithionite (PRIDHAM[8]) gave stable colours, yellow grey with hexoses and sepia with pentoses, thus differing slightly from the colours on acetate-buffered Kieselguhr G. The sprayed (4 directions) plates were heated at 125° for 15 min.

TABLE I

TYPICAL $R_F$ VALUES FOR EIGHT MONOSACCHARIDES USING A 20 × 20 cm PLATE AND SOLVENT 1

| *Sugars* | $R_F \times 100$ | |
|---|---|---|
| | *Single development* | *Double development* |
| Galactose | 10 | 18 |
| Glucose | 14 | 26 |
| Fructose | 20 | 34 |
| Arabinose | 25 | 42 |
| Xylose | 38 | 60[a] |
| Ribose | 39 | 62[a] |
| Fucose | 55 | 77 |
| Rhamnose | 80 | 97 |

[a] Xylose and ribose do not separate.

*Int. J. Biochem.*, 1972, **3**, 33–38. [Scientechnica (Publishers) Ltd.] 

# ON THE APPLICATION TO URINE OF A PHLOROGLUCINOL METHOD FOR MEASURING TOTAL ALDOPENTOSE

D. J. BELL, R. E. CULL,* AND M. Q-K. TALUKDER†

Department of Physiology, The University Medical School, Edinburgh

(*Received* 30 *Sept.*, 1971)

ABSTRACT

1. A phloroglucinol reagent applied to clarified urine is compared with thin-layer chromatography on de-ionized urine in measuring endogenous urinary free aldopentose. The former method gives somewhat high results.
2. In health the excretion of endogenous urinary free aldopentoses is time-dependent.

UNTIL the meticulous work of Date (1958a), who identified and measured D-xylose and L-arabinose in human urine including 2 fasting samples from the same subject, it had not been suspected that urinary aldopentoses had any origin other than alimentary. Later (Date, 1966) it was shown that arabinose, ribose, and xylose were all consistently excreted, in quite regular amounts, in the urine of healthy subjects who had fasted overnight; he also showed that abnormally high fasting rates of excretion of these sugars occur in patients suffering from diabetes mellitus, hepatic cirrhosis, or hyperthyroidism. These sugars must have endogenous sources.

Date's (1958b) procedure involves a tedious two-dimensional paper chromatographic separation which occupies 5 days. Two of us have devised a combination of 2 rapid thin-layer separations (Bell and Talukder, 1970; Talukder, 1971) which enables measurements of the 3 aldopentoses in de-ionized urine with a recovery of 93 per cent (Bell and Talukder, 1972). We wished, however, to have a test, applicable to urine directly, by which the total aldopentose level could be rapidly assessed.

Direct methods for measuring aldopentoses seem of necessity to be based on acid decomposition of the sugars, leading to furfural and other products which form coloured products with aromatic bases or phenols. Such methods usually react also with pentuloses and uronic acids, both of which can be present in varying amounts in urine. Moreover, the hexoses, which are also present even in the fasting state, yield small amounts of furfural because of the slight decomposition of the hydroxymethyl furfural formed in the acidic reagents (cf. Bell, 1966).

The reaction of pentoses with hydrochloric acid and phloroglucinol (Tollens's reagent) as carried out by Dische and Borenfreund (1957) seemed to us to offer a possible means to achieve our end, because free D-glucuronic acid (or its lactone) gives only about 6 per cent of the colour developed by a molar equivalent of aldopentoses, and pentuloses give virtually no colour. According to these authors, glucuronosides (the menthyl derivative only was examined) give a 'negative' reading (*see below*). The small amount of colour developed by glucuronic acid/lactone was important in our application to urine, since Nir, Sicé, and Ivery (1966) and Matsunaga, Imanari, and Tamura (1970) have, by specific methods, shown that this carbohydrate is normally excreted over a range averaging 30 µg. per minute; the

* Present address: Royal Infirmary, Edinburgh.

† Present address: Royal Hospital for Sick Children, Glasgow.

*$R_F$ values*

Using a 20 cm plate, $R_F$ values were markedly increased after a second development with either solvent 1 or 2 allowing quantitative measurements of fucose as well as of galactose, glucose, fructose and arabinose. Rhamnose runs with the solvent front ($R_F$ 0.97); it cannot be satisfactorily measured under these conditions especially if pentuloses, which are very fast running, are present. This system will not separate xylose from ribose (Table I).

On a 35 cm plate and with two developments with solvent 3 the eight monosaccharides were clearly separated as shown by the $R_F$ values (Table II). With this procedure however not more than 15 $\mu$g each of xylose and ribose can be separated quantitatively .The longer plate has other disadvantages because each run takes $3^3/_4$ h and with intermediate drying the whole operation occupies 9 h. Moreover the plate is cumbersome for routine clinical laboratory work. But on occasion, this procedure could be of value for quantitative measurements of the relatively large amounts of galactose, glucose and fructose found free in biological fluids (see $R_F$ values in Table II).

*Quantitative applications*

The procedure was identical with that used by BELL AND TALUKDER[1]. Equal areas of the adsorbent containing the coloured spots were scraped off, the colour extracted by shaking with 3 ml of a mixture of methanol (90 ml) and 5 ml of 1 % (w/v) aqueous stannous chloride. After centrifuging down solids the extinction at

TABLE II

TYPICAL $R_F$ VALUES FOR EIGHT MONOSACCHARIDES ON A 20 × 35 cm PLATE: DEVELOPED TWICE WITH MIXTURE 3

| *Sugars* | $R_F \times 100$[a] |
|---|---|
| Galactose | 28 |
| Glucose | 42 |
| Fructose | 53 |
| Arabinose | 61 |
| Xylose | 77 |
| Ribose | 83 |
| Fucose | 91 |
| Rhamnose | 98 |

[a] All sugars are clearly separated from each other.

TABLE III

TYPICAL EXTINCTIONS FOR FUCOSE AND TWO ALDOPENTOSES STAINED BY PRIDHAM'S REAGENT

| *Amount* ($\mu$g) | *Extinction* | | |
|---|---|---|---|
| | *Fucose* | *Xylose* | *Ribose* |
| 5 | 0.03 | 0.035 | 0.035 |
| 10 | 0.065 | 0.075 | 0.07 |
| 20 | 0.125 | 0.14 | 0.14 |
| 40 | 0.24 | 0.275 | 0.28 |
| 80 | 0.48 | 0.55 | 0.55 |

o nm was measured on an SP 500 spectrophotometer against a blank identically epared from an equivalent area of adsorbent free from sugar spots. Table III shows pical extinctions in the range of 5 to 80 $\mu$g for fucose and also for xylose and ribose n separately under the same conditions.

*etermination of fucose and xylose in the same sample*

This requires two plates, 20 × 20 cm; one is buffered with sodium dihydrogen iosphate (plate A), as described and the other with 0.03 *M* sodium acetate[1] (plate B). late A is developed twice with solvent 1 and the amounts of fucose and (ribose + ylose) determined. Plate B is developed twice with ethyl acetate–propan-2-ol–water :2:1)[1] and ribose determined; the amount of ribose is then subtracted from the ylose + ribose) value obtained from plate A since both pentoses yield the same nount of colour, weight for weight (see Table III).

CKNOWLEDGEMENTS

The author thanks Dr. D. J. BELL for his encouragement and advice. This work as financially supported by the Medical Research Council (grant to D.J.B.) and the overnment of East Pakistan.

EFERENCES

D. J. BELL AND M. Q-K. TALUKDER, *J. Chromatogr.*, 49 (1970) 469.
S. M. PARTRIDGE, *Biochem. J.*, 42 (1948) 238.
K. HOTTA AND M. KUROKAWA, *Anal. Biochem.*, 26 (1968) 472.
M. LATO, B. BRUNELLI, G. CIUFFINI AND I. MEZZETTI, *J. Chromatogr.*, 39 (1969) 407.
M. MURAKAMI AND R. J. WINZLER, *J. Chromatogr.*, 28 (1967) 344.
YU. S. OVODOV, E. V. EVTUSHENKO, V. E. VASKOVSKY, G. OVODOVA AND T. F. SOLOVEVA, *J. Chromatogr.*, 26 (1967) 111.
D. J. BELL, *J. Chem. Soc.*, (1966) 1638.
J. B. PRIDHAM, *Anal. Chem.*, 28 (1956) 1967.

former authors find glucuronosides at about twice this amount.

This paper compares a direct application to clarified urine of the Dische and Borenfreund (1957) reagent with results obtained by thin-layer chromatography (TLC) on the de-ionized fluid; it also indicates situations where the phloroglucinol method is not satisfactory. In an attempt to simplify both types of analysis we examined the possibility of absorbing the urinary sugars by a special charcoal (Hughes and Whelan, 1958). Although this method gives excellent results on the mg. scale, at the µg. level (at which the sugars occur in urine) recovery was always less than 80 per cent. It was partly this regular loss of aldopentose which led two of us to investigate TLC methods.

## EXPERIMENTAL

### Subjects

The subjects were healthy male and female volunteers, who were between the ages of 21 to 31 years except 2 males who were below and above 60 years.

### Dietary Regimens

The subject completed his evening meal by 7.00 p.m. No fruits, cereals, or vegetables (except potatoes) were allowed. Beer, cider, and wine, all of which contain free aldopentoses, were forbidden.

### Urine Collection

Collections of specimens were timed and measured, and if not analysed immediately were stored in presence of toluene at −20° C.

### Analyses

1. *Thin-layer* (Bell and Talukder, 1970; Talukder, 1971)

The urine was treated with ion-exchange resin columns by a procedure analogous to that described by White and Hess (1955) which, after various trials of other possibilities, proved completely satisfactory, giving a recovery of 93 per cent of the total aldopentoses. The de-ionized urine and washings from the columns were concentrated below 40° C. to a suitable volume before applying appropriate amounts to the chromatographic plates.

2. *By Acid-phloroglucinol* (Dische and Borenfreund, 1957)

This method depends on measuring the difference between the extinction ($E$) at 552 nm. and 510 nm. When untreated normal urine is heated with the reagent a dark brown colour develops and no reading can be obtained. The following procedure gave sufficiently satisfactory results (cf. *Table I*): to urine (10 ml.) was added glacial acetic acid (3 drops) and 'activated charcoal' 100 mg. (Bell, 1967) and the whole was shaken for 20 minutes to remove pigments and aromatic compounds. A filtered or centrifuged colourless sample (0·40 ml.) was then treated exactly as described by Dische and Borenfreund (1957). Experiments showed that as much as 1000 µg. per ml. of glucose caused no spectrophotometric interference when $E$ was read at 552 nm. and 510 nm.

3. *Attempts to absorb Urinary Sugars on 'Ultrasorb' Charcoal*

The charcoal, 'Ultrasorb SC 120/240' (British Carbonorite Union Co., Grays, Essex) was shown by Hughes and Whelan (1958) to effect quantitative absorption (with subsequent elution) of monosaccharides when working on the mg. scale. However, at the µg. level, when analyses both by the phloroglucinol method and by TLC were examined, recoveries were only of the order of 70–80 per cent.

## RESULTS AND DISCUSSION

### Phloroglucinol and Thin-layer Results Compared

*Table I* shows that results by phloroglucinol are approximately 7 µg. per minute higher than those by TLC when the latter are corrected for 93 per cent recovery. Urine de-ionization and TLC eliminate glucuronic acid, glucuronosides, sugar phosphates, etc., as well as bases. We do not know whether sugar phosphates occur in urine, although this is a possibility. In our TLC both pentuloses and neutral glucuronolactone are widely separated from the aldopentose regions; both pentuloses and the lactone appear in irregular amounts (by visual inspection only) so that we are uncertain as to the nature of the 'extra' phloroglucinol-reacting material, which may well be glucuronolactone.

Very high phloroglucinol values were found in single instances of 3 females (Cal, McK, and Cur, *Table I*), from whom specimens were taken at different times and places. It is believed that women excrete uronic acids and uronosides in an irregular manner, as measured by methods less specific than those of Nir and others (1966) and by Matsunaga

and others (1970), and that this may be a reflection of their hormonic state. However, if female values greater than 40 µg. per minute are viewed with suspicion, the phloroglucinol method affords a rapid assessment of aldopentose excretion in healthy subjects.

### The Mode of Excretion of Urinary Total Aldopentose

From our observations on healthy fasting males (females were not considered because of the obvious variations in their phloroglucinol reactants), in an individual the total

*Table I.*—Comparison of Direct Analyses by Phloroglucinol and by TLC of De-ionized Urine (µg. per minute)

| Subject | Apparent Aldopentose by Phloroglucinol | True Aldopentose by TLC | Δ |
|---|---|---|---|
| Males | | | |
| McN | 36, 35 | 28, 28 | +8, +7 |
| Bel | 46, 24 | 40, 25 | +6, −1 |
| Tal (m) | 36, 25 | 26, 17 | +10, +8 |
| Pal | 34, 35 | 29, 28 | +5, +7 |
| McA | 35, lost | 25, — | +10, — |
| Mean | | | +6·7 |
| Females | | | |
| Law | 34, 29 | 27, 24 | +7, +5 |
| Tal (f) | 25, 25 | 26, 28 | +1, +3 |
| Nay | 27, 30 | 20, 27 | +7, +3 |
| Cal | 21, 55 | 31, 34 | −10, +21 |
| McK | 31, 50 | 24, 27 | +7, +13 |
| Cur | 33, 48 | 28, 28 | +5, +20 |
| Mean | | | +6·9 |

TLC values are corrected for 93 per cent recovery. All subjects were effectively fasting. Two samples were obtained from each subject on different days.

*Table II.*—The Rates of Excretion of Phloroglucinol-reacting Material by 9 Healthy Males

| Subjects | Excretion Rates (µg. per minute as aldopentose) | Means |
|---|---|---|
| Lei | 12 18F 18FE | 16 |
| Bas | 18 18F 12FE | 16 |
| Cur | 24 24F 18FE | 22 |
| Bel | 30 25 29 30 25 23 | 27 |
| Lit | 30 24F 36FE | 30 |
| Dou | 24 43F 36FE | 34 |
| Tar | 43 36F 43FE | 41 |
| Kan | 51 45F 51FE | 49 |
| Cul | 61 50 47 54 63 44 | 53 |

Each value was obtained on a different day. 'F' indicates that the value was obtained after the fasting subject had consumed 250 g. of lean ham and then rested for 6 hours during which time urine was collected. 'FE' indicates that the subject consumed the ham, rested for 0·5 hour, then walked on a treadmill at 6·4 km. per hour for 2 hours. The individuals are arranged in order of their mean excretions.

The observed differences between individuals are significant ($P<0.01$), but comparing the different treatments (fasting, fed, and fed+exercise) the differences are insignificant (analysis of variance).

*Table III.*—Rates of True Total Aldopentose Excretion (μg. per minute) measured by TLC in 3 Healthy Males during 24 Hours

| Subject | Periods | | | Mean Over 48 Hours | By Phloroglucinol Method |
|---|---|---|---|---|---|
| | 11.00 p.m.–7.00 a.m. | 7.00 a.m.–3.00 p.m. | 3.00 p.m.–11.00 p.m. | | |
| Pas | 36·5 | 32·5 | 37·0 | 35·3 | — |
| Bel | 28·0 | 27·0 | 22·5 | 25·8 | 35·0 |
| Tal | 20·0 | 20·5 | 27·0 | 22·5 | 30·5 |

Each value represents the mean of 2 separate experiments carried out on different days. The subjects consumed no pentose or pentosan.

*Table IV.*—Water-diuresis Experiments on 3 Fasting Males and Effect of Drinking 1·5 l. Cider on 1 of These

| Subject | | Tal (Drank water, 1·4 l.) | | Bel (Drank water, 1·7 l.) | | | Cul (Drank water, 1·5 l.) | | | Cul (Drank cider, 1·5 l.) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Time (hours) after Drinking | Urine Volume (ml. per minute) | Total Pentose excreted | | Urine Volume (ml. per minute) | Total Pentose excreted | | Urine Volume (ml. per minute) | Total Pentose excreted | | Urine Volume (ml. per minute) | Total Pentose excreted | |
| | | μg. per ml. | μg. per minute | | μg. per ml. | μg. per minute | | μg. per ml. | μg. per minute | | μg. per ml. | μg. per minute |
| 1 | 1·94 | 10·0 | 19 | 0·46 | 55 | 25 | 0·58 | 94·0 | 53 | 0·75 | | 55 |
| 2 | 9·75 | 1·7 | 17 | 1·02 | 24 | 24 | 6·52 | 8·5 | 56 | 0·50 | | 40 |
| 3 | 7·25 | 2·3 | 17 | 3·56 | 9 | 32 | 12·16 | 4·5 | 55 | 4·20 | | 83 |
| 4 | 1·08 | 14·0 | 15 | 3·95 | 7 | 28 | — | — | — | 9·00 | | 225 |
| 5 | 1·56 | 10·0 | 16 | 2·36 | 10 | 24 | — | — | — | 13·80 | | 385 |
| 6 | 1·75 | 9·0 | 16 | — | — | — | — | — | — | — | | — |
| Mean rates (μg. per minute) | | | 16·7 | | | 26·6 | | | 54·6 | | | — |
| Rates from *Table II* | | | — | | | 27·0 | | | 53·0 | | | — |

All analyses were by the phloroglucinol method. Each subject fasted for 12 hours before commencing the experiment. The cider (1·5 l.) contained approximately 1·5 g. of pentose(s).

aldopentose appeared to be excreted at a fairly steady rate (*see Table II*), although there were marked variations between our subjects. This variation between individuals is very probably due to non-aldopentose-reacting substances, because TLC measurements of true aldopentose in de-ionized urine showed that differences between individuals were much less marked (*Table III*). Moreover, the *Table III* subjects showed quite regular rates of excretion during the three 8-hour periods examined.

That the aldopentose excretion rate is in fact virtually independent of urine volume was shown by a water-diuresis experiment involving 3 fasting healthy males (*Table IV*) using the phloroglucinol method. It may be noted that 2 subjects (Bel and Cul), on whom previous repeated measurements had been made, again showed individual rates very close to those previously observed in the fasting state (*Table II*).

### 'Alimentary Pentosuria'

Johnstone (1906) first observed that consumption of fruit or beverages derived therefrom resulted qualitatively in excretion of pentoses which could be detected by the original Tollens's test. He distinguished this pentosuria from 'essential pentosuria', which is now known to be genetically determined and is better termed 'L-xylulosuria'. By the

*Table V.*—Typical Results* (obtained by the Phloroglucinol Method) in Experiments on Inducing Alimentary Pentosuria

| Dietary State | Material consumed | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Apple Juice (1 l.) | | | | | Apples (2) | | Beer (1 pt.) |
| | *Subject* | | | | | *Subject* | | *Subject* |
| | L | M | N | O | P | Q | Q | Q |
| Fasting | 23 | 61 | 27 | 20 | 18 | 43 | 68 | 54 |
| Fed | 84 | 112 | 112 | 135 | 190 | 157 | 151 | 87 |
| Difference (Δ) | +61 | +61 | +85 | +115 | +172 | +114 | +83 | +33 |

* Results are expressed as μg. aldopentose per minute.

Fasting subjects consumed the materials stated and collected urine for the subsequent 4 hours. The apple juice was the commercial beverage 'Shloer'.

*Table VI.*—Typical Examples of Urines which contained Phloroglucinol-interfering Chromogens not removed by Acid and Charcoal

| Patient | Day Post-operative | Treatment of Specimen | Spectrophotometric Extinctions | | | Apparent Aldopentose (μg. per min.) |
|---|---|---|---|---|---|---|
| | | | 552 nm. | 510 nm. | Δ | |
| Hul | 1 | Undiluted | 1·20 | 1·30 | −0·10 | 'Negative' |
| | 5 | Undiluted | 0·49 | 0·33 | 0·16 | 25 |
| His | 1 | Undiluted | 0·90 | 0·99 | −0·09 | 'Negative' |
| | 1 | Diluted ×3 | 0·20 | 0·16 | 0·04 | ~18 |
| | 5 | Undiluted | 0·56 | 0·37 | 0·19 | 32 |
| Pur | 1 | Undiluted | 1·30 | 1·58 | −0·28 | 'Negative' |
| | 1 | Diluted ×3 | 0·50 | 0·51 | −0·01 | 'Negative' |
| | 5 | Diluted ×3 | 0·57 | 0·29 | 0·28 | 48 |

The experimental data were obtained on urines from three patients who had undergone open-heart surgery. *E* were measured on a 1-cm. light path.

phloroglucinol method we have demonstrated alimentary pentosuria in a number of volunteers, who consumed apples, apple juice (*Table V*), and cider (*Table IV*).

### Pathological Urines

The spectrophotometry of the method of Dische and Borenfreund (1957) depends on measuring the difference between extinctions of the reacted analytical solutions at 552 nm. and 510 nm.; this procedure gives excellent results with artificial mixtures of sugars and gives acceptable approximations with clarified urines from healthy subjects. *Table VI* illustrates that, even after clarification, some pathological urines can contain substances yielding a very high extinction at 510 nm., which can be sometimes greater than that at 552 nm. We consider that any analysis showing $E_{510}$ greater than 0·50, or greater than 50 per cent of $E_{552}$ should be rejected.

The nature(s) of the interfering substances is not known (cf. Dische and Borenfreund (1957), who examined a number of carbohydrate derivatives). Diseased or hospitalized patients are likely to receive therapeutic drugs which may well be excreted as acidic glucuronosides. Dische and Borenfreund (1957) observed that the one substance of this class which they tested gave a ' negative ' spectrophotometric reading. Further work is required before pathological urines can be reliably examined by the phloroglucinol method; this will certainly necessitate a preliminary de-ionization.

## SUMMARY

1. Compared with the TLC method applied to de-ionized urine, which measures true free aldopentose, the phloroglucinol reagent with clarified urine gives results which are on average 7 μg. per minute higher.

2. Endogenous urinary total free aldopentose is excreted constantly with respect to time and not to urine volume.

3. The phloroglucinol method, as used by us, cannot be relied upon when applied to pathological specimens. With healthy subjects it provides a means of rapidly assessing the approximate aldopentose concentration in urine unless, as occasionally in females, unusually high figures are obtained.

### Acknowledgements

The authors are indebted for financial support to the Medical Research Council (D. J. B.) and to the Government of Bangladesh (M. Q-K. T.). Thanks are due to Mr. D. E. M. Taylor, F.R.C.S.E., who arranged the collection of numerous urines from surgical patients.

## REFERENCES

Bell, D. J. (1966), ' Spectrophotometric microdeterminations of aldoses with *p*-amino benzoic acid ', *J. chem. Soc. C*, 1638–1640.

Bell, D. J. (1967), ' Some monosaccharides in hen's urine ', *Comp. Biochem. Physiol.*, **20,** 523–534.

Bell, D. J., and Talukder, M. Q-K. (1970), ' Thin-layer quantitative chromatography of arabinose, ribose, and xylose in the presence of other sugars ', *J. Chromat.*, **49,** 469–472.

Bell, D. J., and Talukder, M. Q-K. (1972), manuscript in preparation.

Date, J. W. (1958a), ' Quantitative determination of some carbohydrates in normal urine ', *Scand. J. clin. Lab. Invest.*, **10,** 155–162.

Date, J. W. (1958b), ' A quantitative method for estimation of some carbohydrates on filter-paper ', *Scand. J. clin. Lab. Invest.*, **10,** 149–154.

Date, J. W. (1966), ' The excretion of lactose and some monosaccharides in the urine in certain pathological conditions ', *Scand. J. clin. Lab. Invest.*, **18,** 45–50.

Dische, Z., and Borenfreund, E. (1957), ' A new colour reaction for the determination of aldopentose in presence of other saccharides ', *Biochim. biophys. Acta*, **23,** 639–642.

Hughes, R. C., and Whelan, W. J. (1958), ' Removal of electrolytes from sugar solutions ', *Chemy Ind.*, 884–885.

Johnstone, R. W. (1906), ' Pentosuria, chronic and alimentary ', *Edinb. med. J.* **20,** 138–148.

Matsunaga, I., Imanari, T., and Tamura, Z. (1970), ' Simultaneous determination of urinary uronic acids and saccharic acid by gas chromatography ', *Chem. pharm. Bull., Tokyo*, **18,** 2535–2543.

Nir, I., Sicé, J., and Ivery, D. (1966), ' Urinary excretion of D-glucuronic acid and derivatives in man ', *Clin. chim. Acta*, **14,** 756–759.

Talukder, M. Q-K. (1971), ' A rapid quantitative thin-layer chromatographic separation of fucose from other neutral monosaccharides. An application to separate other sugars of physiological interest ', *J. Chromat.*, **57,** 391–395.

White, A. A., and Hess, W. C. (1955), ' Paper chromatographic detection of sugars in normal and dystrophic human urines ', *Archs Biochem. Biophys.*, **64,** 57–66.

*Key Word Index:* Urine, endogenous aldopentose, phloroglucinol, thin-layer restrictions, D-xylose, L-arabinose.

Printed in Great Britain by Henry Ling Ltd., a subsidiary of John Wright & Sons Ltd., at the Dorset Press, Dorchester