of pectinase (Wright, 1961*a*) can be related to the nature of the solid plant particles ingested and utilized by the organism.

SUMMARY

1. Extracts prepared from *Epidinium ecaudatum* by two disintegration procedures contain α-galactosidase and isomaltase activities. Nearly all of the α-galactosidase is extracted by water.

2. The α-galactosidase hydrolyses galactosylgalactosylglycerol faster than it does melibiose and hydrolyses other compounds containing α-(1→6)-linked galactose more slowly than melibiose. 4-α-Galactosylgalactose is also hydrolysed but melibiitol is scarcely attacked and methyl β-L-arabinopyranoside not at all.

3. Galactose is liberated from intact clover galactosyl-lipids, although no lipase activity could be demonstrated in the extracts. β-Galactosylglycerol is not hydrolysed by the epidinial extracts.

4. The α-galactosidase has optimum activity over the pH range 5·0–5·5 and temperature range 38–55°, and acts as a transferase in the presence of 2% of melibiose, galactose or galactosylgalactosylglycerol.

5. The isomaltase, which appears to be a separate enzyme, has optimum activity at pH 6·0.

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The Correlation of Gas–Liquid Chromatographic Behaviour and Structure of Steroids

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The persistent need for microanalytical methods in steroid biochemistry has stimulated many recent applications of gas–liquid chromatography in this field (Horning, VandenHeuvel & Creech, 1963). These have been aided by technical improvements, such as the development of highly sensitive detectors and the use of low concentrations of stationary phase. Silicone-polymer phases of the types introduced by Horning and co-workers (VandenHeuvel, Sweeley & Horning, 1960; VandenHeuvel, Haahti & Horning, 1961*a*) permit the convenient chromatography of a wide variety of steroids, including sterols and other hydroxy steroids that have unduly long retention times on polyester phases. Correlations between retention behaviour and structure have been established by Clayton (1961*a*, *b*, 1962) for a large number of sterol methyl ethers (C_{19}–C_{29}). The 'group retention factor' (Clayton, 1961*a*, *b*) for a particular substituent in a steroid is the relative change in retention accompanying its introduction. In general, the analytical utility of retention factors relies on their substantial independence of molecular weight.

The present paper surveys regularities observed for a number of steroids (C_{19}, C_{21}, C_{24}, C_{27} and C_{28}) with the silicone phases SE-30 and QF-1, principally at 200°. Knights & Thomas (1962*a*, *b*) have

reported useful correlations for a variety of compounds with the QF-1 stationary phase at 250°, and regularities observed with SE-30 at 220° have been described by Tsuda, Ikekawa, Sato, Tanaka & Hasegawa (1962).

The principal aim of the present investigation was to assess the regularities achieved in the gas–liquid chromatography of steroids under standard conditions, and so to assist the identification of steroids isolated in microgram quantities from biological samples. Part of the work has been briefly reported (Brooks & Hanaineh, 1962).

MATERIALS AND METHODS

Steroids. Most of the steroids were obtained commercially or were generously provided by Professor W. Klyne (M.R.C. Steroid Reference Collection); others were kindly given as specified in the footnotes to Table 1. Androst-4-ene-3,11,17-trione and 11β-hydroxyandrost-4-ene-3,17-dione were prepared by the oxidation of cortisone and hydrocortisone respectively (Brooks & Norymberski, 1953). Solutions were made in AnalaR chloroform, usually at concentrations of about 2 mg./ml., and suitable portions were mixed with cholestane for chromatography. The quantity of steroid injected was generally 0·1–1·0 μg.

Apparatus. The Pye Argon Chromatograph was modified to permit convenient injection of solutions. Glass column tubes (134 cm. long, internal diam. 3·5–4·0 mm.) were fitted with a side arm 1 in. below the top for admission of the argon supply, and injections (0·1–2 μl.) were made with a 10 μl. syringe (Hamilton Co. Inc., Whittier, Calif., U.S.A.) through a silicone-rubber 'blind hole' stopper that closed the upper end of the tube. When fully inserted for injection the point of the syringe needle was 1 in. below the side arm. The upper portion of the tube projected from the Chromatograph housing, and was packed only with a loose plug of glass wool. It was enclosed by an aluminium block that incorporated a 300w heating element controlled by a variable transformer. The block temperature was normally kept 25–35° above the main column temperature. Standard conditions were as follows: column temperature, 200±3° (or 225±3°); block temperature, 230±5° (or 250±5°); argon flow rate, 30 ml./min. at outlet (inlet pressure 10–15 lb./in.^{2}); nominal detector voltage, 1250 v; sensitivity setting, 10. The detector was the standard Lovelock argon ionization type, fitted with a ^{90}Sr source.

Preparation of columns. The silicone polymer SE-30 (General Electric Co., Schenectady, N.Y., U.S.A.) and the fluoroalkylsilicone polymer QF-1–0065 (viscosity, 10000 centistokes) (Dow–Corning Corp. Inc.; Midland Silicones Ltd., London) were kindly provided by Dr E. C. Horning.

Column packings were prepared on the support Gas-Chrom P (Applied Science Laboratories Inc., State College, Pennsylvania, U.S.A.) (100–140 mesh for SE-30, 80–100 mesh for QF-1) that had been acid-washed and treated with 5% (v/v) dichlorodimethylsilane in toluene by the procedure of Horning, essentially as described by Sjövall, Meloni & Turner (1961). The lower concentration of dichlorodimethylsilane advised by Holmes & Stack (1962) was not tried. The coating with stationary phase was also based on Horning's technique, as follows: 20 g. of silicone-treated support was suspended in 100 ml. of 1% (w/v) SE-30 in toluene (or QF-1 in acetone), and a gentle vacuum was applied to remove occluded air. After 15 min. the suspension was poured in one lot on to a Buchner funnel, with gentle suction which was released as soon as the bulk of the solution had been filtered. The moist support was transferred to a filter paper, and after being dried in air it was finally dried in the oven at 80°. Column packings so prepared contained approx. 1% (w/v) of stationary phase: samples of the packings used in this work were examined by Soxhlet extraction and found to contain respectively 1·0% (SE-30) and 1·1% (QF-1) of stationary phase.

Glass columns, and glass wool used for packing, were treated with 5% (v/v) dichlorodimethylsilane in toluene, washed with toluene and methanol, and dried before use. Columns were packed by gradual addition of the coated support and repeated tapping; the uppermost inch or two was packed after applying argon pressure (20 lb./in.^{2}) to ensure that undue compaction would not occur in the Chromatograph. The column was not packed above the top of the column heater present in the standard Chromatograph. Before any freshly packed column was used in the Chromatograph it was heated to 300° (SE-30) or 250° (QF-1) in a slow stream of argon for 12–24 hr. to remove volatile products. This process was necessary to avoid contamination of the detector and to ensure that a stable recorder base-line would be secured in the Chromatograph.

Use of columns. All the results described in the present paper were determined with the same pair of columns. The SE-30 column was also used over the same period for more than 1000 chromatograms of other steroid samples, mainly extracts of serum cholesterol. At intervals of 1–2 months the topmost inch of the column packing and the glass wool above it were replaced by fresh materials: this restored sensitivity and reduced 'tailing' caused by the accumulation of decomposed products at the top of the column. The QF-1 column was used almost exclusively for the present work and showed no deterioration over several months. Reproducibility of results from one column to another was not checked with QF-1, except that many of the results were in general agreement with those obtained by one of us (C.J.W.B.) using a QF-1 column in Dr Horning's laboratory (cf. Table 9). With SE-30, preliminary measurements of retention data were carried out on a separately prepared column and showed no appreciable differences from the results given in the present paper.

Determination of retention data. Measurements of retention times were made between the first displacement of the recorder pen after the injection and the point corresponding to the peak of the response to the steroid. Steroids giving asymmetric peaks are indicated in the Tables. The recorded response to the injection was observed 20–25 sec. after the moment of injection and coincided with the return of the outlet flow rate from an elevated level (due to the pressure wave from the evaporation of chloroform) to 30 ml./min. Cholestane (or in a few instances a secondary standard sample) was included in all solutions for injection, and the retention times were expressed as ratios relative to cholestane.

Reproducibility of data. The reproducibility of relative retention values measured at intervals over several months was good for most compounds: variations noted for some di- and tri-ketones were evidently due to their susceptibility to minor temperature changes (cf. Table 9). The fol-

lowing data are illustrative: listed in order are the mean relative retention values (Table 1), S.D. and number of determinations (in parentheses).

(1) On SE-30: 5α-pregnane, 0·148, 0·001 (8); 5α-pregnan-20-one, 0·278, 0·002 (10); 5β-pregnan-3α-ol, 0·283, 0·003 (5); androst-4-ene-3,17-dione, 0·452, 0·003 (9); 3α-hydroxy-5α-androstan-17-one, 0·336, 0·002 (7); 5α-pregnane-3β,20α-diol, 0·652, 0·004 (4); 5α-pregnane-3,11,20-trione, 0·884, 0·002 (7).

(2) On QF-1: 5α-pregnane, 0·206, 0·003 (13); 5α-androstan-3-one, 0·805, 0·003 (14); 5α-androstan-17β-ol, 0·388, 0·005 (7); 5α-pregnane-3,20-dione, 6·30, 0·032 (6); 17β-hydroxy-5α-androstan-3-one, 2·630, 0·017 (11); 5β-pregnane-3α,20β-diol, 1·806, 0·027 (7); androst-4-ene-3,11,17-trione, 12·64, 0·28 (7).

The efficiency of the SE-30 column was about 3000 theoretical plates for cholestane, 1700 for progesterone; neither value changed during 3 months' use. The QF-1 column was much less efficient: values of 1200–1500 were found for ketones, but cholestane gave values as low as 600.

RESULTS AND DISCUSSION

Table 1 summarizes the retention data observed, relative to cholestane, with each of the two columns (stationary phases SE-30 and QF-1) under standard conditions at 200°. Before the results are examined in terms of 'group retention factors' as defined by Clayton (1961*a*, *b*), the following comments are pertinent to indicate the main features of the two silicones used as stationary phases. The almost non-polar character of SE-30 is strikingly shown by the high affinity it shows for the steroid skeleton: cholestane is retained for 30 min. as compared with 6 min. on QF-1. Moreover, the retention of an oxygenated steroid on SE-30 is, to a first approximation, dependent only on its molecular weight and on the number of oxygen functions; their alcoholic or ketonic nature and their situation (with a few exceptions) are of secondary influence. The greater selectivity resulting from the polar structure of QF-1 is shown by the marked variations in retention of functional group isomers and by the much stronger retention of ketones than of alcohols.

Behaviour of steroids epimeric at $C_{(5)}$. It was expected that these different properties of SE-30 and QF-1 would be least apparent in the separation factor for 5α- and 5β-steroids, since the reactivity and accessibility of functional groups at positions 11, 17 or 20 (and of a carbonyl group at position 3) are not grossly affected by the change in nuclear configuration at $C_{(5)}$. The data for 25 pairs of saturated steroids in Table 2 bear out this view: for the pairs 1–13, comprising steroids unsubstituted in ring A and 3-oxo steroids, a mean separation factor (i.e. relative retention) of 1·12 (S.D. less than 0·03) is observed on both columns. A very similar factor (1·10) has been found on SE-30 at 225°. These results are in good agreement with the values of 1·13 (on SE-30 at 210°), from the data of VandenHeuvel, Sjövall & Horning (1961*b*), and 1·1 (on QF-1 at 250°) found by Knights & Thomas (1962*b*). On SE-30, 3α- and 3β-hydroxyl substituents have a scarcely discernible influence (pairs 14–19: 1·11, S.D. 0·02), but on QF-1 factors of 0·98 and 1·28 respectively are observed in the presence of these groups. This ensues from the preferential retention of equatorial alcohols by QF-1, as discussed below with reference to Table 4. It is notable that, on QF-1, acetylation of the 3-hydroxyl group distinctly diminishes this selectivity. [In this respect our results differ from those observed at 250° by Knights & Thomas (1962*b*).] On the other hand, on SE-30, acetylation leads to a marked divergence of retention factors. These observations again illustrate the different character of the two stationary phases, SE-30 responding essentially to molecular skeletal size and shape, and QF-1 interacting selectively with substituent groups. The elution of 3α-hydroxy-5α-androstan-17-one acetate before its 5β-isomer on a polyester column (Lipsky & Landowne, 1961) may now be construed as a result of selective retention of the equatorial acetoxyl group in the latter compound.

The results in Table 2 also show that no significant difference is observed between the retention factors for androstane, pregnane, cholane and cholestane derivatives, in agreement with the more limited data of Knights & Thomas (1962*b*). We have studied a wide range of molecular weights in one series of compounds, giving retention factors (on SE-30 at 200° and 225°) for the introduction of the 3β-hydroxyl group (Table 3). Because of the variety of functional groups present, the results are simultaneously a test of vicinal interactions; nevertheless, it is clear that neither the molecular weights nor vicinal effects seriously impair the constancy of the retention factor for the introduction of the 3β-hydroxyl group with SE-30. The mean values observed are in reasonable agreement with the comparable data for cholestan-3β-ol given by VandenHeuvel *et al.* (1961*a*, *b*). Distinctly lower retention factors are recorded by Sweeley & Lo Chang (1961) and Bloomfield (1962), who used Chromosorb W as support material rather than Gas-Chrom P.

Functional-group retention factors. The main correlations arising from the data of Table 1 are summarized in Table 4 in the form of retention factors for the introduction of hydroxyl and oxo groups. Less than half the data were derived from direct comparisons of monofunctional with parent compounds; in the remainder, the presence of other substituents is indicated where they appear to have a marked effect on the retention factors. Compounds with more obvious interactions

Table 1. *Relative retention data of steroids at* 200°

Gifts of steroids are acknowledged individually in the footnotes. Relative retention values (cholestane = 1·00) are means of at least two determinations on each column and are cited to three decimal places to avoid rounding-off errors in the calculation of retention factors. Retention time of cholestane: 30–33 min. on SE-30, 5·5–6·0 min. on QF-1. Asymmetric peaks are indicated by asterisks.

	Relative retention	
	SE-30	QF-1
Androstanes		
5α-Androstane†	0·078	—
5α-Androstan-3-one	0·175	0·805
5α-Androstan-17-one†	0·161	0·622
5α-Androstan-3β-ol†	0·163	0·427
5α-Androstan-17α-ol†	0·163	0·368
5α-Androstan-17β-ol†	0·166	0·388
5α-Androstane-3,17-dione	0·366	4·649
3α-Hydroxy-5α-androstan-17-one	0·336	1·957
3β-Hydroxy-5α-androstan-17-one	0·338	2·249
17β-Hydroxy-5α-androstan-3-one	0·386	2·630
5α-Androstane-3β,17β-diol	0·353	1·344
5α-Androstane-3,11,17-trione	0·475	9·036
5β-Androstane†	0·071	—
5β-Androstan-3α-ol†	0·147	0·388
5β-Androstane-3,17-dione	0·328	4·306
3α-Hydroxy-5β-androstan-17-one	0·306	2·051
17β-Hydroxy-5β-androstan-3-one	0·344	2·422
5β-Androstane-3α,17β-diol	0·322	1·202
5β-Androstane-3,11,17-trione‡	0·410	8·043
3α-Hydroxy-5β-androstane-11,17-dione‡	0·392	3·984
5α-Androst-2-en-17-one†	0·155	0·631
Androst-4-en-3-one†	0·221	1·287
Androst-4-ene-3,17-dione	0·452	7·247
17α-Hydroxyandrost-4-en-3-one§	0·478	3·893
17β-Hydroxyandrost-4-en-3-one	0·483	4·144
Androst-4-ene-3,6,17-trione	0·642	—
Androst-4-ene-3,11,17-trione	0·555	12·64
11β-Hydroxyandrost-4-ene-3,17-dione	0·822	—
Androst-5-en-3β-ol†	0·163	0·396
3β-Hydroxyandrost-5-en-17-one	0·332	1·990
Androst-5-en-17β-ol	0·167	0·374
Androst-5-ene-3β,17β-diol‡	0·353	1·242
Pregnanes		
5α-Pregnane‖	0·148	0·206
5α-Pregnan-3-one†	0·335	1·381
5α-Pregnan-11-one†	0·201	0·536
5α-Pregnan-20-one†	0·278	0·861
5α-Pregnan-3α-ol†	0·304	0·647
5α-Pregnan-3β-ol†	0·311	0·733
5α-Pregnan-20α-ol†	0·305	0·642
5α-Pregnan-20β-ol†	0·282	0·566
5α-Pregnane-3,20-dione	0·636	6·295
3β-Hydroxy-5α-pregnan-20-one	0·593	3·143
20β-Hydroxy-5α-pregnan-3-one	0·644	3·837
5α-Pregnane-3β,20α-diol	0·652	2·268
5α-Pregnane-3β,20β-diol	0·598	2·010
5α-Pregnane-3,11,20-trione	0·884	15·37
3β-Hydroxy-5α-pregnane-11,20-dione	0·856	8·396
Pregnanes (*cont.*)		
5β-Pregnane†	0·134	0·181
5β-Pregnan-20-one†	0·251	0·779
5β-Pregnan-3α-ol†	0·283	0·653
5β-Pregnan-3β-ol†	0·279	0·574
5β-Pregnane-3,20-dione	0·568	5·754
3α-Hydroxy-5β-pregnan-20-one	0·531	2·821
3β-Hydroxy-5β-pregnan-20-one	0·523	2·486
5β-Pregnane-3α,20α-diol	0·589	2·065
5β-Pregnane-3α,20β-diol	0·544	1·806
5β-Pregnane-3,11,20-trione‡	0·769	13·38
5α-Pregnan-2-en-20-one†	0·272	0·876
5α-Pregn-2-ene-11,20-dione§	0·396	2·330
11α-Hydroxy-5α-pregn-2-en-20-one§	0·484	2·166
16α-Methyl-5α-pregn-2-en-20-one§	0·275	0·832
16α-Methyl-5α-pregn-2-ene-11,20-dione§	0·387	2·132
Pregn-4-en-3-one†	0·420	2·191
Pregn-4-ene-3,20-dione	0·791	9·936
Pregn-4-ene-3,6,20-trione	1·173	—
11α-Hydroxypregn-4-ene-3,20-dione	1·510	—
11β-Hydroxypregn-4-ene-3,20-dione	1·469	—
3β-Hydroxypregn-5-en-20-one	0·585	2·831
Cholanes		
5α-Cholane¶	0·403	0·475
5α-Cholan-12-one¶	0·721	1·857
5α-Cholan-12α-ol¶	0·706	1·096
5β-Cholane¶	0·365	0·431
5β-Cholan-12-one¶	0·648	1·581
5β-Cholan-12α-ol¶	0·618	0·918
Cholestanes		
5α-Cholestane	1·000	1·000
5α-Cholestan-3-one	2·291	6·953
5α-Cholestan-6-one	2·049	—
5α-Cholestan-3α-ol	2·120	3·237
5α-Cholestan-3β-ol	2·135	3·662
5α-Cholestane-3,6-dione	4·223*	—
5β-Cholestane	0·903	0·897
5β-Cholestan-3-one‖	2·055	6·277
5β-Cholestan-3α-ol‖	1·918	3·240
5β-Cholestan-3β-ol††	1·892	2·829
5α-Cholest-2-ene	—	1·006
Cholest-4-en-3-one	2·891*	11·25
Cholest-4-ene-3,6-dione	4·414*	—
Cholest-5-ene	0·999	0·967
Cholest-5-en-3-one	—	11·18
Cholest-5-en-3β-ol	2·120	3·327

† Provided by Professor W. Klyne (London).
‡ Provided by Dr J. K. Norymberski and Dr E. Menini (Sheffield).
§ Provided by Dr C. L. Hewett and Dr G. F. Woods (Organon Laboratories).
‖ Provided by Dr E. C. Horning and Dr W. J. A. VandenHeuvel (Houston, Texas).
¶ Provided by Dr M. Martin-Smith and Dr M. Alauddin (Glasgow).
†† Provided by Dr R. P. Cook (Dundee).

between substituents have been excluded. The principal features of the results are as follows.

(1) On SE-30, the retention factors for hydroxyl substitution at position 3α or 3β in both 5α- and 5β-steroids are all very similar, whereas on QF-1 the equatorial hydroxy steroids (3α-hydroxy 5β-steroids and 3β-hydroxy 5α-steroids) show markedly longer retention than their axial epimers, and can be separated therefrom.

(2) The 17α-, 17β- and 20α-hydroxyl retention factors are hardly distinguishable from one another (or from axial 3-hydroxyl) on either phase.

(3) The sterically hindered 11α- and 11β-hydroxy steroids, and to a lesser extent the 20β-hydroxy steroids, show distinctly lower retention factors; 20-epimers are resolvable even on SE-30.

(4) On both phases the retention factors for ketones diminish in the sequence: Δ^4-3, 3, 17, 20, 11; the separations are small on SE-30 but are greatly enhanced on QF-1, evidently through a combination of polar and steric effects. Thus the relative retention factor of the Δ^4-3-oxo group is increased fourfold, that of the 3-oxo group threefold, and that of the 11-oxo group less than twofold, on QF-1 as compared with SE-30.

(5) Hydroxy and keto steroids have generally similar retentions on SE-30, but the keto steroids are retained relatively more strongly on QF-1, except for the 11-oxo steroids, which are still eluted before the corresponding hydroxy compounds, as on SE-30.

(6) Vicinal effects are more noticeable on QF-1, as expected from its greater reactivity towards functional groups. This is particularly illustrated by the retention factors for 17-oxo and 20-oxo groups in the presence of a 3-oxo group. Consequently SE-30 is more suitable for initial analysis of unknown steroids, and QF-1 for subtler investigations when a tentative structure can be suggested.

The only examples in which androstane and pregnane derivatives have yielded different retention factors are the 11-oxo steroids (Table 5). The apparent distinction may be due to the presence of a 17-oxo group in the androstane derivatives examined, and comparison with compounds lacking this feature is clearly desirable.

Retention factors due to acylation. Though the use of two or more different columns gives the best means of separating mixtures, in practice the changing of columns in a single Chromatograph may be inconvenient. It is then of value to effect chemical modifications, e.g. the selective conversion of hydroxyl groups into, for example, ester or ether groupings, leaving oxo groups unchanged. The changes in retention characteristic of these transformations are useful for the identification as well as for the separation of hydroxy steroids. Thus the retention factor accompanying trifluoroacetylation of 3β-hydroxy 5α-steroids appears to be $0{\cdot}79 \pm 0{\cdot}02$ (on SE-30 at 222°) from four examples cited by VandenHeuvel *et al.* (1961*b*); this derivative permits a decrease in the retention time. Simple acetylation of the 3β-hydroxyl group in the group of normal sterols listed in Table 6 is characterized by a mean retention factor of 1·47 on SE-30 at 225°; variations in substituents as close as $C_{(5)}$ cause only minor changes. The five 4,4-dimethyl sterols examined, on the other hand, give a mean retention factor of 1·36, suggesting that the steric effect of the methyl groups, already well-known in the retention behaviour of the free sterols (Clayton, 1961*a*, *b*, 1962; cf. Brooks, 1963), is enhanced in the bulkier acetates. The retention factors are sufficiently different to be of diagnostic value in the study of naturally occurring sterols.

The influence of the configuration at $C_{(3)}$ and $C_{(5)}$ on the retention factor for acetylation is indicated in Table 7. On the SE-30 column, axial and equatorial 3-hydroxyl substituents are distinguished by acetylation retention factors of 1·45 (S.D. less

Table 2. *Regularities in separation factors for 5α- and 5β-steroids on SE-30 and QF-1 columns at 200°*

The separation factor is the ratio of the retention of the 5α-steroid to that of its 5β-epimer.

5α- and 5β-Epimers	Separation factor SE-30	Separation factor QF-1
1. Androstane	1·11	—
2. Pregnane	1·11	1·14
3. Pregnan-20-one	1·11	1·11
4. Cholane	1·10	1·10
5. Cholan-12-one	1·11	1·19
6. Cholan-12α-ol	1·14	1·17
7. Cholestane	1·11	1·11
8. Androstane-3,17-dione	1·12	1·08
9. 17β-Hydroxyandrostan-3-one	1·12	1·09
10. Androstane-3,11,17-trione	1·16	1·12
11. Pregnane-3,20-dione	1·12	1·09
12. Pregnane-3,11,20-trione	1·15	1·15
13. Cholestan-3-one	1·11	1·11
14. 3α-Hydroxyandrostan-17-one	1·10	0·95
15. Pregnan-3α-ol	1·07	0·99
16. Cholestan-3α-ol	1·11	1·00
17. Pregnan-3β-ol	1·11	1·28
18. 3β-Hydroxypregnan-20-one	1·13	1·26
19. Cholestan-3β-ol	1·13	1·29
20. 3α-Hydroxyandrostan-17-one 3-acetate*	1·02	1·06
21. Pregnan-3α-ol 3-acetate*	1·00	1·08
22. Cholestan-3α-ol 3-acetate*	1·00	1·08
23. Pregnan-3β-ol 3-acetate*	1·23	1·18
24. 3β-Hydroxypregnan-20-one 3-acetate*	1·23	1·17
25. Cholestan-3β-ol 3-acetate*	1·22	1·17

* Compounds 20–25 were prepared by acetylation of compounds 14–19 and were not isolated; the relative retention data are in Table 7.

Table 3. *Changes in relative retention accompanying the introduction of the 3β-hydroxyl group into steroids of the androstane, pregnane, cholestane and ergostane series*

The column used was 1% SE-30 on Gas-Chrom P. The retention data are expressed relative to cholestane (11–12 min. at 225°; 32–33 min. at 200°) = 1·00.

	225°			200°		
	Relative retention		Factor due to 3β-hydroxyl group	Relative retention		Factor due to 3β-hydroxyl group
	3β-Hydroxy steroid	Unsubstituted steroid		3β-Hydroxy steroid	Unsubstituted steroid	
5α-Androstane	—	—	—	0·163	0·078	2·09
5α-Androstan-17-one	0·40	0·20	1·96	0·338	0·161	2·10
5α-Androstan-17β-ol	0·41	0·21	1·93	0·353	0·166	2·12
Androst-5-en-17β-ol	0·40	0·21	1·94	0·353	0·167	2·12
5α-Pregnane	—	—	—	0·311	0·148	2·10
5α-Pregnan-20-one	0·64	0·33	1·96	0·593	0·278	2·13
5α-Pregnan-20α-ol	0·70	0·35	2·00	0·652	0·305	2·14
5α-Pregnan-20β-ol	0·65	0·33	1·95	0·598	0·282	2·12
5β-Pregnane	—	—	—	0·279	0·134	2·09
5β-Pregnan-20-one	0·58	0·30	1·90	0·523	0·251	2·09
5α-Cholestane	1·97	1·00	1·97	2·135	1·000	2·14
5β-Cholestane	1·76	0·91	1·94	1·892	0·903	2·10
Cholest-5-ene	1·96	1·00	1·96	2·120	0·999	2·12
5α-Cholestan-6-one	3·76	1·93	1·95	—	—	—
5α-Ergost-7-ene*	2·94	1·47	2·00	—	—	—
5α-Ergost-8(14)-ene*	2·54	1·28	1·98	—	—	—
5α-Ergosta-7,22-diene*	2·48	1·25	1·98	—	—	—
		Mean values:	1·96 (S.D. 0·03)			2·11 (S.D. 0·02)

* Provided by Dr P. Bladon (Glasgow).

Table 4. *Retention factors due to the introduction of hydroxyl and oxo groups into steroids* (C_{19}–C_{27})

The columns were operated at 200°.

		SE-30			QF-1		
			Retention factor			Retention factor	
Group	Other relevant groups	No. of examples	Range	Mean	No. of examples	Range	Mean
3α-Hydroxyl	5α-Hydrogen	3	2·06–2·12	2·09	3	3·14–3·24	3·18
	5β-Hydrogen	4	2·08–2·12	2·11	3	3·60–3·62	3·61
3β-Hydroxyl	5α-Hydrogen	8	2·09–2·14	2·12	7	3·46–3·66	3·58
	5β-Hydrogen	3	2·09–2·10	2·09	3	3·15–3·19	3·17
	Δ^5	2	2·11–2·12	2·12	2	3·32–3·44	3·38
11α-Hydroxyl	—	2	1·78–1·91	1·84	2	2·47	2·47
11β-Hydroxyl	—	2	1·82–1·86	1·84	2	1·95–2·16	2·06
17α-Hydroxyl	—	2	2·09–2·16	2·13	1	3·02	3·02
17β-Hydroxyl	—	4	2·13–2·19	2·16	3	3·10–3·15	3·13
	3-Oxo	2	2·19–2·20	2·20	2	3·22–3·27	3·24
20α-Hydroxyl	—	3	2·06–2·10	2·08	3	3·10–3·17	3·13
20β-Hydroxyl	—	4	1·91–1·93	1·92	4	2·74–2·78	2·76
3-Oxo	—	6	2·24–2·32	2·28	5	6·71–7·00	6·84
	17- or 20-Oxo	3	2·27–2·29	2·28	3	7·31–7·47	7·39
11-Oxo	—	1	1·36	1·36	1	2·60	2·60
	20-Oxo	5	1·35–1·46	1·41	5	2·33–2·67	2·53
	17-Oxo	4	1·23–1·30	1·27	4	1·75–1·94	1·88
17-Oxo	—	4	2·04–2·08	2·06	3	5·02–5·29	5·19
	3-Oxo	2	2·05–2·09	2·07	2	5·63–5·78	5·70
20-Oxo	—	5	1·87–1·91	1·88	5	4·18–4·33	4·28
	3-Oxo	2	1·88–1·90	1·89	2	4·54–4·56	4·55
Δ^4-3-Oxo	—	6	2·82–2·90	2·85	5	10·64–11·65	11·2

than 0·03) and 1·58 (S.D. less than 0·04) respectively. The situation with QF-1 is more complex, owing to the interplay of steric effects and of the relative affinities of hydroxyl and acetoxyl groups for this phase. Thus the axial 3β-acetoxyl group in 5β-steroids is retained almost as strongly as the equatorial 3β-acetoxyl group in 5α-steroids, and yields a high and characteristic acetylation retention factor of 1·75. The epimeric 3-acetoxy steroids in the 5α-series give practically identical retention factors on this phase. Table 7 shows that the stereochemistry of a 3-hydroxy steroid is assignable with high probability from a knowledge of the retention factors associated with acetylation both on SE-30 and QF-1, together with the relative retention data.

Table 5. *Retention factors due to the introduction of the 11-oxo group into steroids of the androstane and pregnane series*

The columns were operated at 200°.

Parent compound	Retention factor due to 11-oxo group: SE-30	QF-1
5α-Androstane-3,17-dione	1·30	1·94
5β-Androstane-3,17-dione	1·25	1·87
3α-Hydroxy-5β-androstan-17-one	1·28	1·94
Androst-4-ene-3,17-dione	1·23	1·75
5α-Pregnane	1·36	2·60
5α-Pregnane-3,20-dione	1·39	2·44
3β-Hydroxy-5α-pregnan-20-one	1·44	2·67
5β-Pregnane-3,20-dione	1·35	2·33
5α-Pregn-2-en-20-one	1·46	2·66
16α-Methyl-5α-pregn-2-en-20-one	1·41	2·56

Effect of molecular weight. As a corollary of the regularities demonstrated above, particularly with reference to 3-substituents, it is confirmed that retention factors are generally similar in androstane, pregnane, cholane and cholestane derivatives. Within each homologous series the logarithm of the relative retention time is linearly related to the molecular weight, and it thus follows that the various sets of homologues give lines of equal slope. For the majority of the steroids studied these are found to be (*a*) on SE-30 at 200°, $\Delta(\log R_T) = 0{\cdot}010 \times \Delta$(mol.wt.) (on this phase, 11-oxo steroids behave as hydrocarbons of the same molecular weight); (*b*) on QF-1 at 200°, $\Delta(\log R_T) = 0{\cdot}008 \times \Delta$(mol.wt.). These relationships, combined with the appropriate group retention factors, permit the prediction of approximate retention data for a wide variety of steroids. Knights & Thomas (1962*a*) have pointed out the correspondence between the logarithms of relative retention times and the R_M values defined by Bate-Smith & Westall (1950) and extensively applied by Bush (1961) in the paper-chromatographic identification of steroids.

Application of retention data to the analysis of biological extracts. The analytical application of retention factors may be briefly illustrated by an examination of urinary steroid fractions kindly provided by Dr J. K. Norymberski and Dr E. Menini. The fractions had the following expected composition according to the scheme outlined by these authors (Menini & Norymberski, 1962): fraction KA, di- and tri-oxo steroids of the androstane and pregnane series; fraction K, mono- and di-hydroxy-17-oxo steroids of the androstane

Table 6. *Retention factors due to acetylation of the 3β-hydroxyl group of sterols and 4,4-dimethyl sterols*

Chromatography was effected on the SE-30 column at 225°.

	Relative retention data: 3β-Hydroxy steroid	3β-Acetoxy steroid	Factor due to acetylation
Sterols			
Cholestanol	1·97	2·91	1·48
Cholesterol	1·96	2·88	1·47
Cholest-7-enol	2·20	3·26	1·48
Desmosterol	2·17	3·12	1·44
7β-Hydroxycholestanol	3·45	5·20	1·51
7-Oxocholesterol	4·01	5·62	1·40
24-Hydroxycholesterol	3·66	5·42	1·48
Ergosterol	2·45	3·62	1·47
Stigmasterol	2·83	4·20	1·49
		Mean value for normal sterols:	1·47 (S.D. 0·03)
4,4-Dimethyl sterols			
Lanost-7-enol	3·60	4·93	1·37
Lanost-8-enol	3·06	4·16	1·36
Lanosterol	3·40	4·58	1·35
Agnosterol	3·18	4·35	1·37
Dihydroagnosterol	2·88	3·96	1·37
		Mean value for 4,4-dimethyl sterols:	1·36 (S.D. 0·01)

series, derived from urinary 17-hydroxy corticosteroids; fraction KK, fully oxidized compounds corresponding to those in K.

The results observed by chromatography of each fraction on SE-30 and on QF-1 at 200° are summarized in Table 8, and representative chromatograms are shown in Fig. 1. Most of the observed peaks could be tentatively identified by direct comparison with the data for the expected compounds. However, authentic samples of the 3,11β-dihydroxy-5α- and 3,11β-dihydroxy-5β-androstan-17-ones expected in fraction K (from the 5-epimeric tetrahydro derivatives of cortisone and hydrocortisone) were not available. The relative retentions computed for these compounds on SE-30 obtained by using the factor 1·84 for the 11β-

Table 7. *Retention factors due to acetylation of 3-hydroxy steroids*

The acetates were prepared from 50 μg. of hydroxy steroid and were not isolated. The columns were operated at 200°.

	SE-30			QF-1		
	Relative retention data		Factor due to acetylation	Relative retention data		Factor due to acetylation
	Hydroxy steroid	Acetoxy steroid		Hydroxy steroid	Acetoxy steroid	
Axial: 3α-substituted 5α-steroid						
Androstan-17-one	0·336	0·474	1·41	1·957	3·270	1·67
Pregnane	0·304	0·436	1·43	0·647	1·034	1·60
Cholestane	2·120	3·089	1·46	3·237	5·18	1·60
Axial: 3β-substituted 5β-steroid						
Pregnane	0·279	0·406	1·46	0·574	0·994	1·73
Pregnan-20-one	0·523	0·769	1·47	2·486	4·42	1·78
Cholestane	1·892	2·806	1·48	2·829	4·90	1·73
Equatorial: 3β-substituted 5α-steroid						
Pregnane	0·311	0·499	1·61	0·733	1·118	1·60
Pregnan-20-one	0·593	0·943	1·59	3·143	5·16	1·64
Cholestane	2·135	3·411	1·60	3·662	5·718	1·56
Equatorial: 3α-substituted 5β-steroid						
Androstan-17-one	0·306	0·467	1·53	2·051	3·083	1·50
Pregnane	0·283	0·437	1·54	0·653	0·961	1·47
Cholestane	1·918	3·077	1·60	3·240	4·81	1·48

Table 8. *Relative retention data and tentative structures of steroids contained in processed urinary extracts*

The columns were operated at 200°. Parentheses indicate the less probable constituents. The relative peak areas are indicated: w, weak, m, medium, s, strong, vs, very strong.

SE-30		QF-1	
Relative retention	Tentative structure	Relative retention	Tentative structure
Extract KA			
0·31 m	(3ξ-Hydroxy-5β-androstan-17-one)	1·93 vs	(3ξ-Hydroxy-5β-androstan-17-one) (3α-Hydroxy-5α-androstan-17-one) (3β-Hydroxyandrost-5-en-17-one)
0·33 vs	(3ξ-Hydroxy-5α-androstan-17-one) (3ξ-Hydroxy-androst-5-en-17-one) 5β-Androstane-3,17-dione	4·23 vs	5β-Androstane-3,17-dione
0·36 s	5α-Androstane-3,17-dione	4·55 vs	5α-Androstane-3,17-dione
0·57 m	5β-Pregnane-3,20-dione	5·77 m	5β-Pregnane-3,20-dione
0·41 m	5β-Androstane-3,11,17-trione	8·07 w	5β-Androstane-3,11,17-trione
0·47 w	5α-Androstane-3,11,17-trione	9·18 w	5α-Androstane-3,11,17-trione
Extract K			
0·31 m	3ξ-Hydroxy-5β-androstan-17-one	2·05 s	3α-Hydroxy-5β-androstan-17-one 3α-Hydroxy-5α-androstan-17-one 3β-Hydroxyandrost-5-en-17-one
0·335 w	3ξ-Hydroxy-5α-androstan-17-one		
0·54 vs	3ξ,11ξ-Dihydroxy-5β-androstan-17-one	4·0 vs	3α,11β-Dihydroxy-5β-androstan-17-one 3α,11β-Dihydroxy-5α-androstan-17-one
0·60 s	3α,11ξ-Dihydroxy-5α-androstan-17-one 3β,11ξ-Dihydroxy-5α-androstan-17-one	4·8 m	3β,11β-Dihydroxy-5α-androstan-17-one (3α,11α-Dihydroxy-5ξ-androstan-17-one)
Extract KK			
0·33 m	5β-Androstane-3,17-dione	4·2 m	5β-Androstane-3,17-dione
0·36 w	5α-Androstane-3,17-dione	4·6 w	5α-Androstane-3,17-dione
0·40 vs	5β-Androstane-3,11,17-trione	7·8 vs	5β-Androstane-3,11,17-trione
0·47 s	5α-Androstane-3,11,17-trione	8·8 s	5α-Androstane-3,11,17-trione

hydroxyl group are 0·61 and 0·56 respectively, in good agreement with the strong peaks observed at 0·60 and 0·54. The assignment is strengthened by the observation of the two strongest peaks in fraction KK (the oxidation product of fraction K) at 0·47 and 0·40: the conversion of the 11β-hydroxyl group into the 11-oxo group is exceptional in causing a decrease in retention time (cf. Figs. 1*a* and 1*b*), and these values are in good agreement with the recorded values (Table 1) of 0·475 and 0·410 for 5α- and 5β-androstane-3,11,17-trione respectively.

The complementary data for the QF-1 column strengthen some configurational assignments, as shown in Table 8. On the whole, the retention values and the relative peak areas are satisfactorily accommodated by the interpretations given. Rigorous identification of the components of the mixtures would of course demand their isolation; however, the gas–liquid-chromatographic data alone may be sufficiently characteristic in many instances. Thus, in the fully ketonic fraction KK (cf. Figs. 1*b* and 1*c*), the data for the two columns are in complete agreement in confirming the expected composition, and also permit the approximate estimation of the relative proportions of each constituent.

Reliability of retention data. The variability of relative retention data between Laboratories is clearly important, and has been briefly discussed (Brooks, 1963) for SE-30 columns. With QF-1, the data collected in Table 9 indicate fair agreement except for di- and tri-oxo steroids, where the retention relative to cholestane is extremely temperature-dependent. The discrepancies arise from the expected large differences in the temperature–partition coefficient relationships for compounds of widely differing constitution. Retention factors for polyfunctional steroids should accordingly be employed with reference to data for model compounds of related structure, as is customary in the application of other empirical physicochemical information. The separation factors for 5α- and 5β-steroids strikingly illustrate this principle: thus, for SE-30

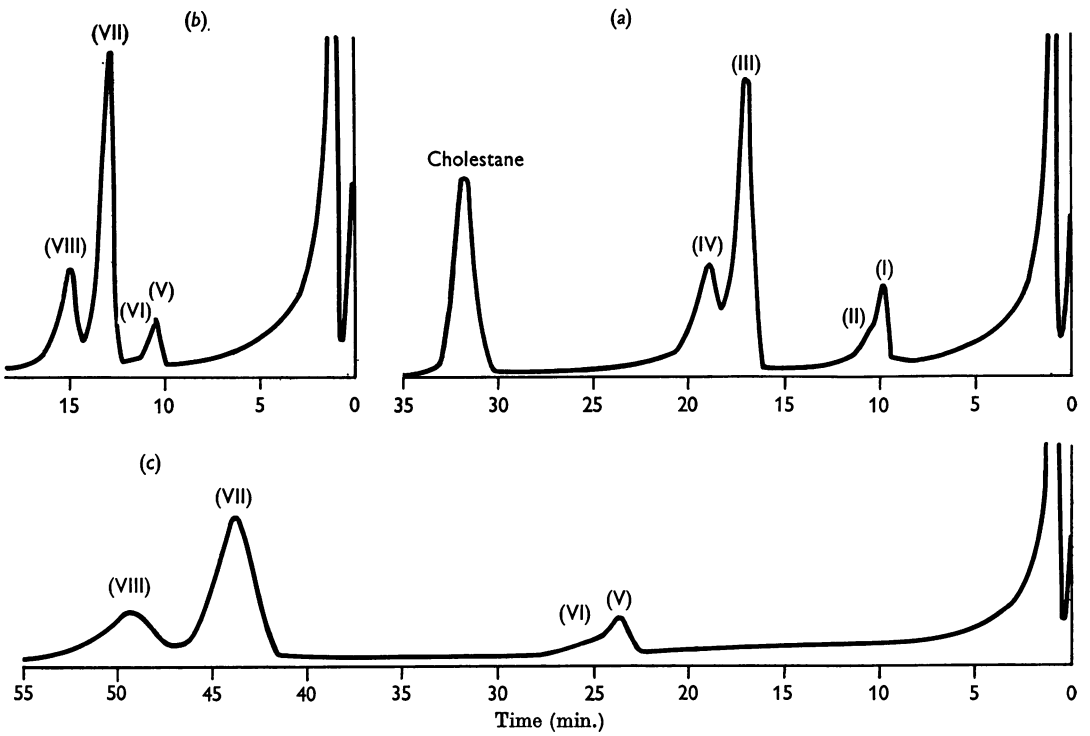

Fig. 1. Gas–liquid-chromatographic separations (at 200°) of keto steroids derived from a urinary extract. Extracts K and KK were prepared as indicated in the text; the conditions for chromatography were as follows: (*a*) extract K (4 μg.) with added cholestane, on SE-30; (*b*) extract KK (5 μg.) on SE-30; (*c*) extract KK (4 μg.) on QF-1. The following are the tentative structures of the separated steroids: (I) 3ξ-hydroxy-5β-androstan-17-one; (II) 3ξ-hydroxy-5α-androstan-17-one; (III) 3ξ,11ξ-dihydroxy-5β-androstan-17-one; (IV) 3ξ,11ξ-dihydroxy-5α-androstan-17-one; (V) 5β-androstane-3,17-dione; (VI) 5α-androstane-3,17-dione; (VII) 5β-androstane-3,11,17-trione; (VIII) 5α-androstane-3,11,17-trione.

Table 9. *Comparative data for the relative retention of steroids on various QF-1 columns*

I, Present work. II, Results obtained by C. J. W. Brooks in Dr E. C. Horning's Laboratory at Bethesda, Md., U.S.A., using a Barber–Colman chromatograph with a 6 ft. column. III, Results obtained by Luukkainen, VandenHeuvel, Haahti & Horning (1961) using a Barber–Colman chromatograph with a 6 ft. column, except for the value for 5α-pregnane-3,20-dione, which was obtained by VandenHeuvel *et al.* (1961*a*). IV, Results obtained by Knights & Thomas (1962*a*, *b*) using a Pye Argon Chromatograph with a 4 ft. column.

	Column and relative retention data				
	1% QF-1 on Gas-Chrom P				6% QF-1 on Celite
	I	I	II	III	IV
Temperature ...	200°	225°	~210°	195°	250°
Steroid					
3α-Hydroxy-5α-androstan-17-one	1·96	—	1·88	—	2·18
5α-Androstane-3,11,17-trione	9·04	7·48	8·04	—	—
5β-Androstane-3,11,17-trione	8·04	6·65	7·12	—	7·41
5α-Pregnane-3β,20β-diol	2·01	1·89	1·88	1·94	2·02
5α-Pregnane-3β,20α-diol	2·26	2·10	2·08	2·16	2·25
3β-Hydroxy-5α-pregnan-20-one	3·14	2·82	2·88	—	—
20β-Hydroxy-5α-pregnan-3-one	3·84	3·43	3·60	—	—
5α-Pregnane-3,20-dione	6·30	5·35	5·72	5·93	5·90
5α-Pregnane-3,11,20-trione	15·4	12·1	13·08	—	12·42
5β-Pregnane-3,20-dione	5·75	4·99	5·32	—	5·37
5β-Pregnane-3,11,20-trione	13·4	10·8	11·52	—	11·10
5α-Cholestan-3β-ol	3·66	—	3·16	3·70	3·03
Cholest-5-en-3β-ol	3·33	—	3·00	3·39	—

at 225°, the factors for six 5α–5β pairs (3, 7, 13, 16, 18 and 19 in Table 2) are within 1% of those at 200°. Similarly on QF-1 the separation factors for the 5α- and 5β-androstanetriones, pregnanediones and pregnanetriones (Table 9) at 225° and (with the Barber–Colman chromatograph) at about 210° are within 2% of the values at 200° given in Table 2, despite the gross changes in retention relative to cholestane.

It is concluded that gas–liquid-chromatographic retention data, when carefully interpreted, permit the convenient and rapid identification of steroids in samples amounting to a few micrograms. Where rigorous characterization is imperative, the isolation of separated steroids may still be required. However, it is arguable that the specificity of an infrared-absorption spectrum could be matched by that of a suitable array of gas–liquid-chromatographic information. Indeed, at the microgram quantity the latter is likely to be considerably more definitive.

SUMMARY

1. Retention data, relative to cholestane, for gas–liquid chromatography of 90 steroids of the androstane, pregnane, cholane and cholestane series have been recorded for two silicone stationary phases, SE-30 and QF-1, on Gas-Chrom P at 200°.

2. A constant separation factor of 1·12 (S.D. less than 0·03) is observed on both phases for 5α- and 5β-epimers of saturated steroids, either bearing no 3-substituent or with a 3-oxo group.

3. Regularities in 'group retention factors' (changes in retention accompanying the introduction of a substituent) have been demonstrated for 3-, 11-, 17- and 20-hydroxyl groups and for 3-, 11-, 17-, 20- and Δ^4-3-oxo groups. The influence of other structural features is indicated.

4. Acetylation retention factors for 3-hydroxy steroids have been correlated with the stereochemistry of rings A and B.

5. Retention data have been applied to the tentative identification of keto steroids derived from urine.

We are indebted to Dr E. C. Horning (Houston, Texas) for his generous guidance, and to Professor E. J. Wayne and Professor R. A. Raphael, F.R.S., for their encouragement. Grateful acknowledgement is also made to the donors of steroids mentioned in the text, and to Dr J. K. Norymberski and Dr E. Menini for allowing us to report results secured with their preparations.

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Ion Transport in Rat-Liver Slices

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The relation between metabolism and ion transport is a subject of general interest. It has been studied in many tissues (Harris, 1960). One of the techniques for the study of ion transport has been to leach out part of the potassium from a tissue. When the tissue is then placed into a favourable environment, potassium reaccumulates. Leaf (1956) showed that cooling led to the entry of sodium and water into, as well as the loss of potassium from, cooled liver and kidney slices. Mudge (1951), using kidney slices, showed that potassium was lost on cooling, and that it reaccumulated when the slices were incubated in warm Ringer solution. Maizels (1951) and Maizels & Remington (1958) have used similar cooling and warming techniques for red cells, liver slices and kidney slices.

However, rat-liver slices, one of the most convenient laboratory preparations, have not given consistent results by this technique. The ion movements produced by cooling and rewarming were often small and slow (Maizels & Rimington, 1958; D. Parsons, personal communication). The present paper describes a procedure that gives consistent and rapid ion transport in rat-liver slices. The results of using these methods to study the effects of drugs and nutritional state on ion transport are reported by Judah & McLean (1962) and by McLean (1963).

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MATERIALS AND METHODS

Preparation of liver slices. Albino rats of 100–200 g. weight were used. All rats were given 20 mg. of α-tocopherol acetate 1 day before they were killed. The cube diet used (MRC 41B cubes) was deficient in vitamin E, and liver slices from animals even mildly deficient in vitamin E show defects of ion transport (McLean, 1960*a*, *b*).

The rats were killed by a blow on the head and exsanguinated, and the livers were rapidly removed. The liver was cut into slices by hand with a long razor blade and a guide similar to that described by McIlwain (1961). Slices 0·3 mm. thick and weighing 100–200 mg. were easily produced in numbers up to 20 per liver.

The slices were put into ice-cold 'saline solution' [containing NaCl (150 mM) and KCl (5 mM)] as soon as they were cut. The slices and saline were kept in a squat beaker, surrounded by crushed ice and water to maintain a low temperature (about 2°). The slices were kept cold for 35 min., by which time 70% of their original potassium content had leaked out. The slices were then distributed to vessels for incubation at 38°.

When oxygen uptake was to be measured as well as slice composition, single slices were put into 3 ml. of Ringer solution in Warburg flasks.

To measure the time-sequence of ion movements, up to three slices were put into 10 ml. of Ringer solution in a 50 ml. conical flask. Slices were withdrawn at intervals from the flask. The flasks were corked and gassed through wide-bore hypodermic needles. A piece of polythene tubing led the gas to the surface of the incubation medium. Ringer phosphate solutions were gassed with oxygen, Ringer bicarbonate solutions with $O_2 + CO_2$ (95:5). Flasks were shaken on a Warburg bath at 80–100 strokes/min.

Incubation solutions. The Ringer phosphate solution with added calcium contained: NaCl (125 mM), KCl (6mM),