column under these circumstances is lower (cf. James & Martin, 1952), but is adequate to separate any three of the four amines provided a suitable liquid phase is chosen (Table 3).

Fig. 6. Analysis of the volatile amines present in *Chenopodium vulvaria* L. Curve A: standard mixture of mono-, di- and tri-methylamines. Curve B: chromatogram of the amines present in an acid extract of the plant, showing the presence of only ammonia and trimethylamine. (Column length, 4 ft.; liquid phase, hendecanol-15% liquid paraffin (v/v); temp., 78.5°; flow rate of nitrogen, 5.7 ml./min.; nitrogen pressure, 80 mm. Hg.)

The lower limit of quantity of amine that can be detected with this technique is 0.3 µg. equiv., i.e. 2 µg. of NH_3 , 4 µg. of monomethylamine, 7 µg. of dimethylamine and 8 µg. of trimethylamine. The maximum amounts of amines that still allow good separations are 160 µg. of NH_3 , 180 µg. of monomethylamine, 180 µg. of dimethylamine and 220 µg. of

trimethylamine, using a 4 ft. 4 mm. internal-diameter column.

The estimation of the amines present in *Chenopodium vulvaria* L. The leaves from four plants of *C. vulvaria* L. (approx. 100 g.) were minced in a Waring Blendor and extracted with 0.001 N-HCl (1 l.). The extract was filtered, centrifuged and then made alkaline with NaOH. Air was drawn through the mixture into a wash bottle containing 0.001 N-HCl. At the end of an hour the wash bottle was removed and its contents evaporated almost to dryness. The solution was made up to 2 ml., and 0.1 ml. portions were taken for analysis. The results are shown in Fig. 6.

SUMMARY

1. A technique for the micro-estimation and separation of ammonia and the three methylamines is described.
2. Some factors influencing the relative positions of amines on gas-liquid partition chromatograms are discussed.
3. A method of applying the amines to the columns starting from a solution of the amine salts is described.
4. All the curves illustrated have been obtained by the use of the automatic recording burette described by James & Martin (1952). Three types of manual microburette are now described, together with appropriate titration procedures.

Our thanks are due to Dr B. T. Cromwell for the gift of the stinking goosefoot. Part of this work (by G.H.S.) was carried out as part of the programme of the Food Investigation Organization of the Department of Scientific and Industrial Research.

REFERENCES

- Cromwell, B. T. (1950). *Biochem. J.* **46**, 578.
 Fuks, N. A. & Rappoport, M. A. (1948). *C.R. Acad. Sci. U.R.S.S.* **60**, no. 7, 1219.
 James, A. T. (1952). *Biochem. J.* **52**, 242.
 James, A. T. & Martin, A. J. P. (1952). *Biochem. J.* **50**, 679.
 Lagerkvist, U. (1950). *Acta chem. scand.* **4**, 543.
 Reay, G. A. (1937). *Rep. Food Invest. Bd., Lond.*, p. 69.

Gas-liquid Partition Chromatography: the Separation of Volatile Aliphatic Amines and of the Homologues of Pyridine

By A. T. JAMES

National Institute for Medical Research, Mill Hill, London, N.W. 7

(Received 1 January 1952)

Previous papers (James & Martin, 1952; James, Martin & Howard Smith, 1952) have described the separation of volatile fatty acids and also of ammonia and the methylamines by means of gas-liquid partition chromatography. This study has now been extended to include higher aliphatic amines and pyridine homologues.

Aliphatic amines

By the use of columns having liquid paraffin as the stationary phase a great many primary, secondary and tertiary aliphatic amines may readily be separated from one another in amounts between 0.3 and 10 µg. equiv. In Fig. 1, curve A shows the

separation of methylamine, ethylamine, *isopropylamine*, *n*-propylamine, *isobutylamine*, *n*-butylamine, *diisopropylamine*, *isoamylamine* and *n*-amylamine at a temperature of 100°. The first few amines are not completely resolved, as the column has been run deliberately at a flow rate higher than that suitable for such separations in order to show as many amines as possible on a reasonable time scale. Diethylamine, *sec*.-butylamine and *isobutylamine* have retention volumes (James & Martin, 1952) so similar that they cannot be resolved on such a 4 ft. column. Curve B (Fig. 1) shows the separation of

crease by a factor of 2.1–2.2 for an increase in chain length of one $-\text{CH}_2$ group (a factor of 1.9–2.0 was found for the volatile fatty acids by James & Martin, 1952). The primary *isoalkylamines* show the same factor, as would be expected. The constancy of this factor shows that the van der Waals forces involved in interaction between the carbon chain of the amines and paraffin liquid phase on the columns increase by a constant amount for each additional $-\text{CH}_2$ group. The fact that the primary *isoalkylamines* are separated by a constant factor (1.3–1.4) from their straight-chain isomers indicates the

Fig. 1. The separation of aliphatic amines at a temperature of 100°, using a 4 ft. column with liquid paraffin as the stationary phase. Curve A: nitrogen pressure, 75 mm. Hg; nitrogen flow rate, 5.7 ml./min. Curve B: nitrogen pressure, 225 mm. Hg; nitrogen flow rate, 18.7 ml./min. For data on behaviour of other amines see text and Table 1.

isoamylamine, *n*-amylamine, *di*-*n*-propylamine, 4-methylpentyl-1-amine (*iso*-hexylamine), *n*-hexylamine, *di*-*sec*.-butylamine, *n*-heptylamine and *di*-*n*-butylamine at 100° and at a higher flow rate than that used to obtain curve A. In this case triethylamine and *isoamylamine* run together as do *di*-*sec*.-butylamine, *diisobutylamine* and *cyclohexylamine*. Tri-*n*-propylamine overlaps *n*-heptylamine. Table 1 gives a list of the retention volumes of the amines relative to that of ethylamine together with their boiling points.

The regularity of behaviour of the aliphatic amines on such paraffin columns is demonstrated by Fig. 2, where the logarithm of the relative retention volume (retention volume of amine/retention volume of ethylamine) is plotted against the number of carbon atoms for a series of primary, secondary and tertiary amines.

The retention volumes (V_R) of the primary straight-chain homologues above ethylamine in-

crease by a factor of 2.1–2.2 for an increase in chain length of one $-\text{CH}_2$ group (a factor of 1.9–2.0 was found for the volatile fatty acids by James & Martin, 1952). The primary *isoalkylamines* show the same factor, as would be expected. The constancy of this factor shows that the van der Waals forces involved in interaction between the carbon chain of the amines and paraffin liquid phase on the columns increase by a constant amount for each additional $-\text{CH}_2$ group. The fact that the primary *isoalkylamines* are separated by a constant factor (1.3–1.4) from their straight-chain isomers indicates the

difference in van der Waals interactions between the groups $-\text{CH}_2$, CH_2 , CH_3 and $-\text{CH}(\text{CH}_3)_2$, and the carbon chains of the paraffin. The poor separation of *sec*.-butylamine and *isobutylamine* (Table 1) and of *di*-*sec*.-butylamine and *diisobutylamine* indicates that there is a much smaller difference of energy of interaction with the liquid phase between the groups $-\text{CH}_2$, $\text{CH}(\text{CH}_3)_2$ and $-\text{CH}(\text{CH}_3)_2$, CH_2 , CH_3 . The same effect is shown with the fatty acids *isovaleric acid* and α -methylbutyric acid (James & Martin, 1952). The difference, however, should be sufficient to allow the two amines to be separated in an 11 ft. column (James & Martin, 1952).

The slope of the line connecting the points for the secondary straight-chain amines is slightly less than that connecting the points for the primary amines (Fig. 2). The slope of the tertiary amine line is markedly less, confirming the suggestion made by James *et al.* (1952) that in series NH_2R , NHR_2 , NR_3 each additional alkyl group contributes less to the

van der Waals forces of interaction with the liquid phase of the column.

A non-polar liquid phase such as paraffin allows no hydrogen bonding between molecules of solute and solvent and so the separations are due solely to differences in the van der Waals forces between the solute and solvent. (This ignores any hydrogen bonding between the solute and the kieselguhr support for the liquid phase, which is, in fact, small.)

Table 1. *Retention volumes of amines relative to that of ethylamine with non-polar and polar liquid phases in the columns at 100°*

(Those amines marked with the same small letters cannot be completely resolved on a 4 ft. column.)

Amine	B.p. (°)	Column liquid phase	
		Paraffin	Lubrol MO
Methylamine	-6.5	0.61	0.68 <i>b</i>
Dimethylamine	7.4	0.93 <i>a</i>	0.81
Ethylamine	16.6	1 <i>a</i>	1
Trimethylamine	3.5	1.17	0.67 <i>b</i>
<i>iso</i> Propylamine	34	1.49	1.42
<i>n</i> -Propylamine	48.7	2.20	2.10 <i>c</i>
Diethylamine	55.5	3.25 <i>d</i>	2.10 <i>c</i>
<i>sec</i> -Butylamine	63	3.57 <i>d</i>	2.8 <i>e</i>
<i>iso</i> Butylamine	68	3.70 <i>d</i>	3.1 <i>e</i>
Ethylenediamine	118	4.65 <i>f</i>	15.5
<i>n</i> -Butylamine	77.8	4.7 <i>f</i>	4.4
Ethanolamine	172.2	5.25	25.8
Diisopropylamine	83	6.8	3.00 <i>e</i>
Triethylamine	89.5	8.6 <i>g</i>	3.1 <i>e</i>
<i>iso</i> Amylamine	95	8.8 <i>g</i>	7.3 <i>h</i>
<i>n</i> -Amylamine	104	10.5	9.7
Di- <i>n</i> -propylamine	110.7	13.2	7.2 <i>h</i>
4-Methylpentyl-1-amine	123.9	17.8	13.8
<i>n</i> -Hexylamine	132.7	22.6	19.6
Di- <i>sec</i> -butylamine	132.0	27.6 <i>i</i>	9.74 <i>j</i>
<i>cyclo</i> Hexylamine	134	28.4 <i>i</i>	25.9
Diisobutylamine	139	30.4 <i>i</i>	10.9 <i>j</i>
<i>n</i> -Heptylamine	158.3	49.5 <i>k</i>	40.05
Tri- <i>n</i> -propylamine	156	49.6 <i>k</i>	15.1
Di- <i>n</i> -butylamine	159	62.1	27.6
Tri- <i>n</i> -butylamine	214	—	85.5
Benzylamine	185	81.0	91.5

Primary and secondary amines contain both active hydrogen atoms ($\cdots\text{H}-\text{N}=\text{}$) and donor atoms ($\cdots\text{N}=\text{}$) capable of forming hydrogen bonds with suitable groups, e.g. the hydroxyl group of an alcohol, the resulting association complexes being of the types (a) $\text{R}_2\text{N}-\text{H}\cdots\text{O}(\text{H})-\text{R}'$ and (b) $\text{R}_3\text{N}\cdots\text{H}-\text{O}-\text{R}$. With solvents such as ethers, which unlike alcohols contain only donor atoms, only one type of hydrogen bonding is possible, namely, (c) $\text{R}_2\text{N}-\text{H}\cdots\text{O}-\text{R}'(\text{R}'')$. Tertiary amines possess no active hydrogen atoms and thus form associated complexes only with alcohols (type (b)) and not with ethers. 'Lubrol MO' (a polyethylene-oxide-long-chain alcohol condensate) probably has the repeating structure

and with few if any hydroxyl groups. Only primary and secondary amines would be expected to show strong hydrogen bonding with this solvent. On this basis it would be expected that primary and

Fig. 2. The relationship between $\log_{10}$ (retention volume of amine/retention volume of ethylamine) and total number of carbon atoms in the amine molecule. $\triangle-\triangle$, primary straight-chain amines; $\circ-\circ$, primary *iso*alkylamines; $\square-\square$, secondary straight-chain amines; $\bullet-\bullet$, tertiary straight-chain amines.

secondary amines would have greater retention volumes on columns having Lubrol MO as the stationary phase than on columns having liquid paraffin as stationary phase, whilst tertiary amines would have similar retention volumes on both

Table 2. *The effect of hydrogen bonding on the corrected retention volumes of primary, secondary and tertiary amines with two different liquid phases on the columns at 100°*

Amine	V_R^0 (ml.)*	
	Paraffin	Lubrol MO
<i>n</i> -Butylamine	90	234
Di- <i>n</i> -propylamine	250	383
Trimethylamine	22	35
Tri- <i>n</i> -propylamine	960	800

* Defined by James & Martin, 1952.

columns (assuming the van der Waals forces to be of the same order in both solvents). Table 2 gives a list of the corrected retention volumes of four representative amines on Lubrol MO and liquid-paraffin columns, showing the expected relationship. The exceptional behaviour of trimethylamine is explicable if it is assumed that the H atoms of the

methyl groups are 'active' enough to allow hydrogen bonding of the type $(\text{CH}_3)_2\text{NCH}_2\text{—H}\cdots\text{O—R}'(\text{R}'')$. This suggestion is supported by a comparison of the boiling points of trimethylamine (3.5°) and trimethylmethane (2-methylpropane) (-10.2°) which suggests that hydrogen bonding of the trimethylamine molecules may occur. The boiling points of triethylamine (89.5°) and triethylmethane (3-ethylpentane) (93.3°) are much closer so that it can be assumed that no hydrogen bonding occurs with amines higher than trimethylamine.

Fig. 3. The relationship between relative retention volumes (retention vol. of amine/retention vol. of ethylamine) for aliphatic amines on columns having stationary liquid phases of (a) liquid paraffin and (b) Lubrol MO, showing the relative speeding up of secondary and tertiary amines on changing from a solvent not allowing, to one allowing, hydrogen bonding. $\triangle$ — $\triangle$, primary straight-chain amines; $\circ$ — $\circ$, primary isoalkylamines; $\square$ — $\square$, secondary straight-chain amines; $\odot$ — $\odot$, secondary isoalkylamines; $\bullet$ — $\bullet$, tertiary straight-chain amines.

The order of hydrogen-bonding strength probably follows that of basicity of the amines (primary and secondary > tertiary) and, on columns having Lubrol MO as the stationary phase, secondary and tertiary bases run more rapidly with respect to a given primary amine than on columns having liquid paraffin as the stationary phase. In Table 1 is shown a comparison of relative retention volumes of a series of 27 amines on both types of column. Fig. 3

shows the relationship between relative retention volumes on the two types of column for a series of primary, secondary and tertiary amines. The values for the three types of amine lie on straight lines showing the expected relationship, namely, slope of the line for tertiary amines greater than that for secondary amines greater than that for primary amines. The contribution to the solvation energy of the amines made by hydrogen bonding is still greater in the case of a diacidic amine such as ethylenediamine so that it runs much more slowly on Lubrol MO (relative $V_R = 15.5$) than on paraffin (relative $V_R = 4.65$). In this case the solvent paraffin is so unlike the pure solute ethylenediamine that the amine (mol.wt. 78) behaves similarly to *n*-butylamine (relative $V_R = 4.7$, b.p. 77.8° , mol.wt. 83). Lubrol MO has solvent properties much more akin to pure ethylenediamine so that the base (b.p. 118°) behaves similarly to 4-methylpentyl-1-amine (relative $V_R = 13.8$, b.p. 123.9° , mol.wt. 101).

Identification of aliphatic amines

This property of hydrogen bonding with the solvent can be used to decide whether a given amine is primary, secondary or tertiary, or diacidic by measuring its relative retention volume on the two types of column and finding on which line of the graph in Fig. 3 the values fall. In addition, where zones of two or more amines of differing basic strength overlap on liquid paraffin columns, the amines can be separated by re-running on Lubrol MO columns. In this way dimethylamine and ethylamine; diethylamine and *iso*- or *sec*-butylamine; ethylenediamine and *n*-butylamine; triethylamine and *iso*amylamine; cyclohexylamine and *diiso*- or *di-sec*-butylamine, can be separated. Of the 27 amines examined only two pairs, *iso*- and *sec*-butylamine; *diiso*- and *di-sec*-butylamine cannot be separated on 4 ft. columns of either solvent.

Table 3. Factors of increase in relative retention volume for an increase of chain length of one —CH_2 group

	Stationary liquid phase	
	Paraffin	Lubrol MO
Primary amines	2.2	2.1
Secondary amines	2.1	1.9-2.0
Tertiary amines	1.80	1.71

In Table 3 the increases in retention volume for an increase of chain length of one —CH_2 group in primary, secondary and tertiary amines are compared for both types of column. The constancy of these factors allows the prediction of position of any primary, secondary or tertiary amine (having straight or branched chains) provided the retention volume of any other amine in the correct homologous series is known.

Pyridine homologues

Fig. 4 (curve A) shows the separation of pyridine, α -picoline, γ -picoline, 2:6-lutidine, 2:4-lutidine and 2:4:6-collidine on a 4 ft. column at 137° using liquid paraffin as the stationary phase. β -Picoline and γ -picoline cannot be separated; the two zones overlap completely. In Table 4 a comparison of the silicone DC550, Lubrol MO and liquid paraffin as stationary phases is made and it can be seen that liquid paraffin is superior, in that it allows a separation of γ -picoline and 2:6-lutidine. The hydrogen-bonding

and 2:4:6-collidine. The 2:6-lutidine fraction contains α -picoline, γ - and/or β -picoline, and 2:6-lutidine; the 2:4-lutidine fraction contains a small amount (approx. 4%) of γ - and/or β -picoline and the 2:4:6-collidine contains a small amount of 2:4-lutidine. The increased width of the collidine zones shows more than one of its isomers to be present. This method of analysis of coal-tar fractions should allow some approach to be made to the standardization of the lutidine and collidine fractions used in paper chromatography, where considerable variations in R_F values are found when different samples are used.

Fig. 4. The separation of pyridine homologues on a 4 ft. column at 137° having liquid paraffin as the stationary phase. (Nitrogen pressure, 19 cm. Hg; nitrogen flow rate, 15 ml./min.) Curve A: bases in order of emergence from the column: pyridine, α -picoline, β - and γ -picoline, 2:6-, 2:4- and 2:5-lutidine, 2:4:6-collidine. Curve B: analysis of a commercial sample of 2:6-lutidine showing the presence of α -picoline, γ - and/or β -picoline and 2:6-lutidine. Curve C: analysis of a commercial sample of 2:4-lutidine showing the presence of γ -picoline. Curve D: analysis of a commercial sample of collidine showing the presence of 2:4-lutidine. In addition, the broadness of the collidine band indicates the presence of two further components.

Table 4. *Relative retention volumes of pyridine homologues*

(Temperature, 137°; values relative to that of pyridine, taken as 1.)

Column liquid phase	α -Picoline	β -Picoline	γ -Picoline	2:6-Lutidine	2:4-Lutidine	2:4:6-Collidine
DC550	1.39	2.3	2.24	1.94	2.82	4.30
Lubrol MO	1.50	2.3	2.34	2.15	3.40	4.85
Liquid paraffin	1.75	2.4	2.31	2.80	4.00	6.35

powers of pyridine, α -picoline, β -picoline and γ -picoline are very similar, so that transferring from a solvent with little to one with high hydrogen-bonding power has little or no effect on their relative position. 2:6-Lutidine, 2:4-lutidine and 2:4:6-collidine probably have a decreased hydrogen-bonding power so that they run more rapidly relative to pyridine on columns allowing hydrogen bonding.

The relative retention volumes of these pyridine homologues may be easily related to those of the aliphatic amines as pyridine has a retention volume relative to ethylamine of 10.5 (at 100°) on paraffin columns, and 10.3 on Lubrol MO columns.

The curves B, C and D (Fig. 4) show the behaviour of commercial samples of 2:6-lutidine, 2:4-lutidine

EXPERIMENTAL

Preparation of columns. The columns were of the size (4 ft. long and 4 mm. internal diameter) described by James & Martin (1952). The kieselguhr used was prepared as described by James *et al.* (1952). The liquid phases of liquid paraffin, 'Lubrol MO' (Imperial Chemical Industries Ltd.) and DC550 (Midland Silicones Ltd.) were added to the kieselguhr in the proportion of 3 g. of liquid phase to 7 g. of kieselguhr.

Procedure. The amines were added to the columns with a micropipette either in ethanolic solution (33%, w/w) or as the pure dry amine. The titrations were carried out using the automatic recording burette of James & Martin (1952) filled with 0.04N-H₂SO₄. The burette has been modified to take two burette tubes held parallel to one another, one containing dilute acid, the other dilute alkali. While one is in operation connected to the titration cell, the other is con-

nected only to its reservoir. In this way either bases or acids may be titrated without having to empty and refill the burette. The indicator used was methyl red in saturated aqueous solution. For further experimental details the two earlier papers should be consulted.

SUMMARY

1. The separation and micro-estimation of a series of aliphatic primary, secondary and tertiary amines is described.

2. A straight-line relationship is obtained when the logarithms of the retention volumes are plotted against the number of carbon atoms for each type of primary, secondary and tertiary amine. By means of this relationship the relative retention volume of any amine may be predicted provided the retention volume of any other member in the correct homologous series is known.

3. The utilization of columns having polar and

non-polar liquid phases to determine whether an unknown amine is primary, secondary or tertiary is described. A plot of the relative retention volumes of primary, secondary and tertiary amines on paraffin against those obtained with 'Lubrol MO' liquid phases shows the values for each type of amine to fall on straight lines of different slope.

4. Out of 27 amines containing from 1 to 12 carbon atoms only two pairs (*iso*- and *sec*.-butylamine; *diiso*- and *di-sec*.-butylamine) cannot be separated on 4 ft. columns.

5. The separation of the homologues of pyridine is described as a suitable method for the approximate standardization of pyridine bases for chromatographic use.

Thanks are due to Dr A. J. P. Martin, F.R.S., for much valuable advice and discussion. Appreciation is also expressed to the National Coal Board (Eastern Region) and Messrs Yorkshire Tar Disillers Ltd. for gifts of pyridine homologues.

REFERENCES

James, A. T. & Martin, A. J. P. (1952). *Biochem. J.* **50**, 679.

James, A. T., Martin, A. J. P. & Smith, G. Howard (1952). *Biochem. J.* **51**, 323.

Studies in Immunochemistry

11. THE ISOLATION AND PROPERTIES OF THE HUMAN BLOOD-GROUP H SUBSTANCE

By E. F. ANNISON AND W. T. J. MORGAN
The Lister Institute of Preventive Medicine, London, S.W. 1

(Received 11 January 1952)

The different sera of human and animal origin which bring about the agglutination of human O erythrocytes strongly, whilst agglutinating cells belonging to other blood groups less readily or not at all, are generally referred to as 'anti-O' sera. The isolation from human red cells of a material possessing the capacity to neutralize the agglutinating action of these sera on O cells was first reported by Hallauer (1934) and subsequently by Stepanov, Kuzin, Markageva & Kosyakov (1940). Witebsky & Klendshoj (1941) described the isolation of a small quantity of serologically active material from the gastric juice of group O persons. The homogeneity of the substances was in no instance established, but the results were important in that they demonstrated that serologically specific materials of carbohydrate nature could be obtained from the erythrocytes and gastric juice of group O persons. Landsteiner & Harte (1941) likewise isolated the so-called O substance from the saliva of individuals

belonging to group O and showed that the material was largely carbohydrate in nature and could not be distinguished chemically from that obtained from the saliva of persons belonging to groups A and B.

Following the demonstration by Morgan & Van Heyningen (1944), that ovarian cyst fluids obtained from women secreting their specific blood-group substances in a water-soluble form are a convenient and potent source of these materials, Morgan & Waddell (1945) isolated from this source a polysaccharide-amino-acid complex which possessed the so-called O specific character. The material obtained was not electrophoretically homogeneous, but nevertheless a preliminary examination of the substance was undertaken and it was found to possess a laevorotation with $[\alpha]_{5461} - 22^\circ$, to contain 5.9% nitrogen and to yield, after acid hydrolysis, 43% of reducing sugars, expressed as glucose, and 33% of hexosamine, as glucosamine base. Hydro-