Davison, P. F., James, D. W. F., Shooter, K. V. & Butler, J. A. V. (1954). *Biochim. biophys. Acta*, **15**, 415.
Johns, E. W. (1960). *J. Chromat.* **5**, 91.
Johns, E. W., Phillips, D. M. P., Simson, P. & Butler, J. A. V. (1960). *Biochem. J.* **77**, 631.
Neelin, J. M. & Connell, G. E. (1959). *Biochim. biophys. Acta*, **31**, 539.
Neelin, J. M. & Neelin, E. M. (1960). *Canad. J. Biochem. Physiol.* **38**, 355.
Phillips, D. M. P. (1958). *Biochem. J.* **68**, 38.
Phillips, D. M. P. & Johns, E. W. (1959). *Biochem. J.* **72**, 538.
Sautière, P. (1959). Doctoral Thesis: Lille University.
Smithies, O. (1955). *Biochem. J.* **61**, 629.
Svensson, H. (1956). *Ciba Foundation Symp., Paper Electrophoresis*, p. 94.

Biochem. J. (1961) **80**, 193

Chromatographic Methods for the Study of Amines from Biological Material

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(*Received* 24 *January* 1961)

Amines occur in most biological fluids and extracts, and considerable work has been done on their isolation, in particular where the amines are of physiological interest, as are the catechol group, and 5-hydroxytryptamine and histamine. The methods used have usually been designed specifically for isolating the amine or group of amines in question, rather than for general application to all amines. The procedures presented here were chosen for their ability to deal with bases in general and for their relative mildness; they are not applicable to amino acids, which have already received adequate attention.

Since biological material usually contains amines in relatively low concentration, chromatography has been widely used to study them. Some of the methods have been adapted from those developed for amino acids; others have been worked out specially for amines (on paper: Bremner & Kenten, 1951; Schwyzer, 1952; Herbst, Keister & Weaver, 1958; on ion-exchange resins: Weber, 1951; Gardell, 1953; Kirshner & Goodall, 1957). Not all of the standard amino acid techniques can be applied to amines: thus electrolytic desalting only works with neutral compounds or ampholytes, and sulphonated polystyrene resins retain amines too firmly for convenient separation, so that rather powerful eluents are needed (Wall, 1953). Weakly acidic resins proved to have the necessary properties to overcome both these difficulties.

In the present work the amines were concentrated from a dilute solution or extract by ion-exchange resins used in the sequence described by Weber (1951), and the mixture of bases, after separation from most of the inorganic material, was resolved by column chromatography on buffered, weakly acidic resin. The fractions were analysed for amines, and those containing the peaks were then desalted on another column of resin in the free acid form. The resulting solution on evaporation to dryness yielded the crystalline amine hydrochlorides. Paper chromatography in five different solvents and paper electrophoresis were used to compare the products thus isolated with known compounds.

MATERIALS AND METHODS

Buffer solutions. Except where otherwise stated, buffer solutions are those of McIlvaine (1921).

Ion-exchange resins. De-Acidite FFX (100-mesh beads, chloride form; Permutit Co. Ltd.). This resin was specially made for the present work, and differs from the commercially available De-Acidite FF by improved chemical stability. It was regenerated by stirring with twice its volume of 5% NaOH, filtering on a sintered funnel, and repeated washing on the filter with 5% NaOH until the filtrate was free of chloride. The resin was then washed with boiled (i.e. CO_2-free) and cooled distilled water until free of alkali. Columns were poured from a slurry of this resin in CO_2-free water. If not used at once, the column was rinsed again before use, since residual traces of alkali and of tertiary amine were leached from the resin on prolonged standing in the free-base form.

Zeo-Karb 226 (50 μ beads, hydrogen form; Permutit Co. Ltd.) was treated as described for the similar resin Amberlite XE-64 by Hirs, Moore & Stein (1953).

Zeo-Karb 226 (100-mesh beads, hydrogen form: Permutit Co. Ltd.) was graded by backwashing (Hamilton, 1958). Coarse and fine fractions, of about 10% of the total amount of resin each, were rejected, and the main fraction was then treated like the 50 μ bead resin. It was buffered by stirring with twice its volume of the appropriate buffer, and solid A.R. NaOH was then added to the stirred suspension of resin until the pH of the supernatant remained constant at the

original value. Columns of the resin were poured from a slurry made up in the same buffer.

Preparation of the amine concentrate. The solution or extract containing the amines, with 0·1% (v/v) of thiodiglycol (di-2-hydroxyethyl sulphide) added as an antoxidant, was passed through a column of De-Acidite FFX in the free-base form at a flow-rate of 20–40 ml./cm.²/min. (manufacturer's recommendation). This displaced the equilibrium between the chloride and hydroxide forms of the resin towards the chloride form by removal of hydroxyl ions, and was achieved, where necessary, by passing compressed N_2 into the reservoir above the column: the apparatus was built to withstand an excess of pressure of about 1 atm.

The effluent, followed by two equal rinses of CO_2-free water, was allowed to flow directly into a stirred suspension of Zeo-Karb 226 (50 μ beads, hydrogen form) in water. A rough calculation indicated the amount of resin needed, allowing for 20% excess. When all the effluent and rinsings had been added, stirring was continued for an hour. The pH of the supernatant was then 6·5.

The Zeo-Karb 226 containing the amines was poured into a column, allowed to drain but not to dry out, and washed with two equal portions of water to remove neutral compounds. The combined organic and inorganic bases were eluted with 0·5 N-HCl, twice the volume at which the acid front emerged being used. The eluate was concentrated by vacuum-distillation at room temperature and evaporated to dryness *in vacuo* over a mixture of flake NaOH and granular $CaCl_2$, giving a concentrate containing amine hydrochlorides and ammonium and metal chlorides. Where the proportion of inorganic material was low, the concentrate was used as such, otherwise it was extracted with three successive portions of boiling 95% ethanol, leaving a residue of inorganic material. The ethanol extract was evaporated to dryness *in vacuo* before the next stage.

Ion-exchange chromatography of the concentrate. Zeo-Karb 226 (100-mesh beads, buffered at pH 7·3) was poured into a column to a depth of 110 cm. The column had a short side arm, closed with a self-sealing plug, near the top. When the top of the bed was level with the side arm the buffer (pH 7·3) was allowed to drain down to the top of the bed, and both the reservoir and the space above the bed were filled with buffer at pH 5·0. A solution of the amine concentrate in this buffer was injected through the plug with a syringe, and elution was allowed to proceed. Optimum conditions were as follows: load about 25 mg. of N/cm.² cross-sectional area, in not more than 2 ml. of buffer/cm.²; flow-rate 10–20 ml./cm.²/hr. At the end of the elution the whole column was at pH 5·0, and the pH of the effluent changed to this value from the initial pH 7·3. Elution volumes of some amines are shown in Table 1.

Detection of amines in the effluent fractions. Primary and secondary amines were determined by the modified Folin method of Blau & Robson (1957), by using 1 ml. of each fraction and adding 4 ml. of 50% ethanol, instead of taking 5 ml. of solution. The ethanol prevented precipitation of the coloured compounds formed from some of the higher-molecular-weight amines. Table 2 gives the colour yields and limits of linearity of some primary and secondary amines.

Table 1. *Elution volumes of some amines in column chromatography*

The amines were chromatographed as described in the text, some singly and others in mixtures. The elution volume is the total volume eluted at the amine peak with in each case a column of 0·8 cm. bore and depth 112·5 cm., filled with Zeo-Karb 226 buffered at pH 7·3, and eluted with buffer pH 5·0. Counting was started after rejection of the first 25 ml. after application of the sample.

Amine	Elution volume (ml.)	Shape of peak	Recovery
Trimethylamine *N*-oxide	12	Very sharp	Quant.
Creatinine	20	Very sharp	Quant.
Tetramethylammonium	79	Sharp	Quant.
Diethylamine	81	Sharp	Quant.
n-Amylamine	109	Sharp	Quant.
*iso*Amylamine	111	Sharp	Quant.
Trimethylamine	118	Sharp	Quant.
Piperidine	128	Sharp	Quant.
n-Butylamine	130	Sharp	Quant.
Pyrrolidine	148	Sharp	Quant.
n-Propylamine	150	Sharp	Quant.
Dimethylamine	162	Sharp	Quant.
Ethylamine	184	Sharp	Quant.
Tyramine	260	Broad	85%
Glucosamine	270	Broad	85%
Methylamine	280	Broad	Quant.
Canavanine	290	Very broad	80%
Ethanolamine	300	Broad	80%
Adrenaline	310	Very broad	Not determined
Arginine	330	Broad	70%
3-Hydroxytyramine	430	Very broad	90%
Noradrenaline	460	Very broad	70%
5-Hydroxytryptamine	465	Very broad	80%
Ammonia	490	Broad	Quant.
pH 5·0 breakthrough	600	—	—

Total N was determined by the micro-Kjeldahl method of Lang (1958); 1 ml. of each fraction was pipetted into a Pyrex tube, and the tubes were heated with the digestion mixture in a square aluminium block drilled with 100 holes.

Desalting of amine solutions. The fractions containing an amine peak were combined, made just acid to Congo red paper and evaporated to dryness *in vacuo*. The residue was dissolved in the minimum volume of 0·025 N-HCl and the solution was injected through a self-sealing plug closing a short side-arm at the top of a column containing Zeo-Karb 226 (100-mesh beads, hydrogen form; column diameter 2·5 cm., bed depth 20 cm.; this column was suitable for desalting about 50 mg. of amine). The whole system including the reservoir was kept filled with 0·025 N-HCl, which was also used for the elution. Small portions of the fractions were withdrawn and analysed for total N as above, and elution was stopped after emergence of the amine, leaving the column ready for desalting once more. Fig. 1 shows an elution curve for a solution containing sodium citrate and piperidine. Where the volume of solution applied was large, or where the proportion of salt was high, it was sometimes necessary to evaporate the fractions containing amine down to small volume and repeat the desalting. The amine peak always emerged at the same volume for a given column. Evaporation of the combined fractions containing the amine left a residue of the crystalline amine hydrochloride.

Table 2. *Colour yields of some amines by the modified Folin method of Blau & Robson* (1957)

Extinction was measured in a Unicam SP. 500 spectrophotometer at a wavelength of 476 mμ in 10 mm. glass cells. The final solution contained 2 ml. of ethanol out of a total of 9 ml.

Amine	Colour yield (extinction units/μmole)	Limiting amount (μmoles)
Adrenaline	0·15	5+
n-Amylamine	0·43	4
*iso*Amylamine	0·43	3
Ammonia	0·23	5+
Arginine	0·39	4
n-Butylamine	0·47	4
1:4-Diaminobutane	0·84	2
1:5-Diaminopentane	0·68	3
Diethanolamine	0·19	3
Diethylamine	0·19	4
Dimethylamine	0·53	4
Ethanolamine	0·40	4
Ethylamine	0·39	4
Glucosamine	0·12	5+
Glycine	0·49	5+
Histamine	0·36	3
Hydroxyproline	0·53	4
5-Hydroxytryptamine	0·38	4
3-Hydroxytyramine	0·38	3
Methylamine	0·42	4
Noradrenaline	0·38	4
Phenethylamine	0·48	2
Piperidine	0·56	4
Piperidine-3-carboxylic acid (pipecolic acid)	0·12	2
Proline	0·49	4
n-Propylamine	0·46	4
Pyrrolidine	0·53	3
Sarcosine	0·59	3
Tryptamine	0·35	6
Tyramine	0·43	3

Paper chromatography. Whatman no. 1 paper was developed by descending chromatography counter to the machine direction, after at least 2 hr. of equilibration in the vapour of the solvent. The following solvent systems were used: butan-1-ol–conc. HCl–water (7:2:1, by vol.; homogeneous; pH 1); butan-1-ol–acetic acid–water (4:1:5, by vol.; two phases; the mixture was refluxed for an hour and left to separate overnight; the lower layer was used for equilibration, and the upper, pH 3, for development); 2-methoxyethanol–propionic acid–water (14:3:3, by vol.; saturated with solid NaCl; homogeneous; pH 2; Herbst *et al.* 1958); butan-1-ol–acetic acid–water–pyridine (15:3:12:10, by vol., homogeneous, pH 5; Waley & Watson, 1953); pyridine–pentan-1-ol–water (3:3:2, by vol.; homogeneous; pH 9). The developed chromatograms were dried in a current of air and sprayed with the following reagents: Folin's amino acid reagent, 1% 1:2-naphthoquinone-4-sulphonic acid (purified sodium salt) followed by saturated $KHCO_3$; ninhydrin–$Cu(NO_3)_2$ (Moffat & Lytle, 1959); alkaline ferricyanide–nitroprusside (Block, Durrum & Zweig, 1955) used without dilution; Ehrlich's reagent (Smith, 1953); diazotized *p*-anisidine (Sanger & Tuppy, 1951) modified by replacing half the *p*-anisidine with sulphanilamide; isatin (Boyarkin, 1956), developing the colours by heating at 100° for 10 min. after spraying; Sakaguchi reagent (Williams, 1951); Jaffé reagent, saturated picric acid followed by N-NaOH; Nessler's reagent (Koch & McMeekin, 1924). The papers were inspected under u.v. light, then hung in an atmosphere saturated with I_2 vapour, and finally sprayed after hanging in a current of air to remove I_2. The I_2 did not affect the colour reactions, except for easily oxidized amines. The R_F values in Table 3 have been related to that of dimethylamine, which was arbitrarily chosen as convenient. This empirically improved their reproducibility, and involved measuring the R_F of dimethylamine in each solvent from at least 30 measurements. 'Corrected solvent fronts' on subsequent chromatograms were drawn from the positions of reference spots of dimethylamine included at either end and in the centre of every sheet, and the R_F values of all the other spots were

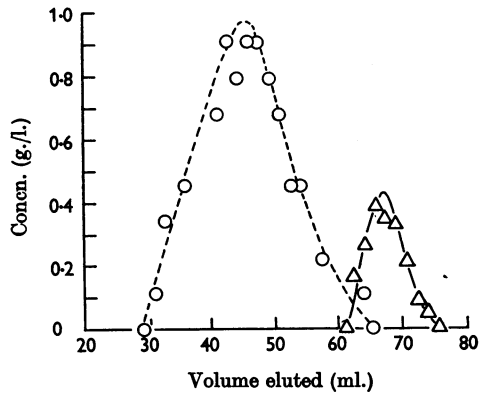

Fig. 1. Elution curve from a solution containing 100 mg. of trisodium citrate (dihydrate) and 5 mg. of piperidine hydrochloride in 3 ml. of water applied to a column (2·4 cm. × 18 cm.) of Zeo-Karb 226 (hydrogen form) and eluted with 0·025 N-HCl. Recoveries were quantitative within experimental error. ○, Sodium; △, piperidine.

Table 3. *Standardized R_F values of amines*

R_F values are related to those of dimethylamine (in bold type), as described in the text. The solvent systems are designated by letters as follows: A, butan-1-ol–acetic acid–water; B, butan-1-ol–acetic acid–water–pyridine; C, 2-methoxyethanol–propionic acid–water; D, pyridine–pentan-1-ol–water; E, butan-1-ol–conc. HCl–water. The spray reagents are abbreviated as follows: I_2, iodine vapour; NQS, Folin's reagent; NCN, ninhydrin–$Cu(NO_3)_2$; FCNP, alkaline ferricyanide–nitroprusside; EHR, Ehrlich's reagent; IS, isatin; UV, fluorescence under u.v. light; NN, diazotized *p*-anisidine–sulphanilamide; JAF, alkaline picric acid; NESS, Nessler's reagent; SAKA, Sakaguchi's reagent; IP, potassium iodoplatinate; for details of solvents and spray reagents see text. Numerical colour values refer to those on the 'Derwent' colour chart (see text and Reio, 1958).

Amines were applied to the paper as 0·5 % solutions of their salts (mainly the hydrochlorides, otherwise the bitartrates and acid citrates) in 50 % ethanol.

Compound	100 R_F					Colours						Other reagents
	A	B	C	D	E	I_2	NQS	NCN	FCNP	EHR	IS	
Acetylcholine	39	45	78	43	46	++	.	.	.	.	.	IP:39 (24)
Adrenaline	39	54	70	65	43	++	4	.	11	.	20	NN:9; UV:+
Agmatine	06	35	42	04	24	+	68	26	22	(2)	26	SAKA:18
4-Amino-5-carbamoyl-glyoxaline	40	55	64	50	34	.	25	70	70 (26)	6	7	NN:65; IP:9
p-Aminobenzoic acid	85	92	82	79	73	+	8	16	.	2	.	UV:+; NN:2
2-Amino-2-hydroxymethyl-propane-1:3-diol (tris)	21	43	67	38	33	.	(34)	.	.	.	.	.
1-Aminopropan-2-ol	34	48	69	33	43	+	31	24	.	.	19	.
N-(3-Aminopropyl)-1:4-diaminobutane (spermidine)	05	18	27	02	08	++	29	24	.	.	24	IP:71
p-Aminosalicylic acid	43	56	65	41	72	++	21	9	46	1	8	NN:57; UV:+
Ammonia*	22	42	56	32	24	.	68	.	72	(40)	.	NESS:58
*iso*Amylamine	69	77	84	68	87	+	69	24	.	(2)	11	IP:72
n-Amylamine	68	83	87	71	92	+	27	26	.	(1)	12	.
Aniline	94	98†	90	90†	79	+	21	.	.	5	.	.
Arginine	14	26	38	12	20	.	70	26	22	(1)	19	SAKA:18
Atropine	69	81	92	68	86	+++	.	.	.	.	.	IP:35
Benzylamine	63	76	84	68	74	+	33	24	.	(1)	22	IP:72
Betaine	27	32	59	18	44	+	.	.	.	.	.	IP:(24)
Brucine	61	70	77	60	64	++	7	.	.	.	.	IP:35
n-Butylamine	61	73	83	64	78	++	69	24	.	(1)	24	.
Caffeine	93	86	85	64	54	++	.	.	.	.	.	UV:+
Canavanine	14	19	36	07	16	.	(52)	26	22	.	24	SAKA:18
N-(2-Chloroethyl)dibenzyl-amine (Dibenamine)	86	90	78	93	96	++	.	.	.	.	.	IP:(26)
Choline	31	37	70	26	45	++	.	.	.	.	.	IP:(71)
Citrulline	16	26	48	11	28	.	69	26	.	1	24	.
Creatine	37	54	57	40	30	+	.	.	22	.	.	.
Creatinine	36	51	71	40	37	.	(26)	.	72 (14)	.	.	JAF:9
Cystamine‡	14	35	44	25	18	+++	31	22	.	(1)	24	IP:1
1:4-Dianinobutane	11	25	38	10	18	++	29	26	.	(1)	68	IP:72
1:2-Dianinoethane	13	27	33	04	14	+	18	24	.	(47)	63	IP:72
1:5-Diaminopentane	12	30	44	12	19	++	31	26	.	(2)	41	IP:72
1:3-Diaminopropane	10	26	36	22	15	+	68	24	.	.	19	IP:72
NN'-Di-(3-aminopropyl)-1:4-diaminobutane (spermine)	02	02	12	43	05	.	26	.	.	.	.	IP:71
Diethanolamine	25	44	70	12	41	+	9	.	.	.	.	UV:+
Diethylamine	55	62	83	58	73	++	17	.	.	.	69	.
2-Dimethylaminoethanol	32	44	71	43	45	+	.	.	.	.	.	.
N-Dimethylaniline	87	99†	97	.†	84	++	.	.	.	.	.	.
Dimethylamine (reference compound)	**33**	**43**	**70**	**30**	**47**	++	11	26	.	.	12	.
NN-Dimethylguanidine	46	56	76	55§ 36	57	++	.	.	17 (24)	.	.	.
NN-Dimethylurea	70	73	76	62	74	+	.	.	.	(4)‖	.	.
NN'-Dimethylurea	62	66	74	61	83	+	.	.	.	(4)‖	.	.
Diphenylamine	97	99	99	93	99	+++	41	.	.	46	3	UV:+
Ethanolamine	26	41	65	23	35	.	33	26	.	(2)	14	.
Ethylamine	39	50	72	36	50	++	68	24	.	.	2	.
Ethyleneimine	42	56	74	10	52	+	26	24	.	.	9	.
Glucosamine	16	36	49	33	20	.	39	.	.	(1)	19	UV:+
Glycocyamine	30	28	56	16	42	.	.	.	14 (22)	.	.	.
Guanidine	38	47	67	36	47	++	.	.	7	.	.	UV:+; SAKA:18
Harmaline	75	80	80	87	83	++	48 (51)	4	51 (45)	1	52	UV:+; NN:9; IP:65
Hexamethylenetetramine	31	40	63	29	81	++	.	.	.	.	.	IP:18
*cyclo*Hexylamine	65	75	84	67	88	++	52	.	.	.	2	.
Histamine	13	33	34	31	18	+	70	24	.	.	24	IP:72; NN:8
Histidine	12	24	25	14	13	+	69	24	18	(1)	24	NN:8
5-Hydroxytryptamine	44	64	75	65	39	++	69	18	44	26	12	NN:12
3-Hydroxytyramine	40	61	78	64	44	+++	51	18	18	(1)	8	NN:8
Indole	96	99	92	94	99	++	21	.	.	23	.	NN:7
Lysine	10	20	37	07	17	+	26	26	.	(1)	35	.
Methylamine	30	40	65	31	38	+	68	26	.	(2)	13	.

Table 3 (*cont.*)

Compound	100 R_F A	B	C	D	E	Colours I_2	NQS	NCN	FCNP	EHR	IS	Other reagents
2-Methylaminoethanol	31	45	70	11	45	+	15	.	.	.	19	.
N-Methylaniline	81	96	90	93	83	++	15	.	.	.	.	.
Methylguanidine	46	55	73	52§ 38	57	+	.	.	14	.	.	SAKA:18
2-Methylindole	95	99	90	93	99	++	19	.	.	21	.	UV:+; NN:6
3-Methyindole (skatole)	97	99	91	95	99	++	70	.	.	24	.	UV:+
N-Methylpyridine	37	39	72	26	44	+++	.	.	.	.	.	UV:+; IP:38
Methylurea	61	64	74	58	73	++	18	.	57	2	57	UV:+
α-Naphthylamine	96	99	87	90	86	++	19	.	.	3	.	UV:+; NN:12
β-Naphthylamine	96	99	85	95	86	++	20	.	.	4	.	UV:+; NN:9
Nicotine	54	61	81	87	33	++	.	.	.	.	.	IP:39
Noradrenaline	30	50	66	59	31	++	69	5	11	.	20	UV:+; NN:10
Ornithine	09	19	30	07	13	+	31	26	.	(1)	35	.
Phenethylamine	69	76	84	69	83	+	24	71	.	(1)	26	IP:71
Piperidine	53	61	77	53	65	+	14	.	.	.	47	.
Piperidine-2-carboxylic acid (pipecolic acid)	38	44	63	28	62	+	17	.	.	.	37	.
n-Propylamine	50	63	82	59	64	+	26	26	.	(2)	.	.
Pyridine	20	44†	60	30†	44	.	.	.	.	.	.	IP:38
Pyridine-2-carboxylic acid (nicotinic acid)	75	71	78	39	41	+	.	.	.	.	.	.
Pyrrolidine	43	53	74	47	58	++	13	.	.	.	41	.
Quinine	77	91	97	80	74	++	.	.	.	.	.	UV:+; IP:28
Quinoline	90	43	86	92	69	++	.	.	.	.	.	IP:38
Sparteine	66	93	95	71	61	++	.	.	.	.	.	IP:31
Trigonelline	26	32	54	18	38	+++	.	.	.	.	.	IP:34
Tri-*n*-amylamine	95	96†	98	95†	98	++	.	.	.	.	.	IP:71
Trimethylamine	30	67	65	55	50	++	.	69	.	.	.	.
Trimethylamine *N*-oxide	43	49	76	23	58	++	11	.	.	.	21	.
Trimethylvinylammonium (neurine)	37	41	77	26	47	+++	.	.	.	.	.	IP:34
Tri-*n*-propylamine	83	89†	98	.†	94	++	.	.	.	.	.	IP:72
Tryptamine	62	76	81	67	64	+++	24	18	.	22	19	UV:+
Tyramine	55	72	80	67	62	++	68	69	.	(1)	70	NN:9
Urea	45	49	67	42	56	.	(11)	.	16	1 (48)	.	.
Yohimbine	78	84	95	86	89	+++	44	.	.	.	.	UV:+; IP:63

* Ammonia could only be detected in high concentrations.

† Pyridine, some tertiary and some aromatic amines are difficult to detect, except at high concentrations, after being run in solvents containing pyridine.

‡ Cysteamine gave the same colours and had the same R_F values as cystamine, and it is assumed that it was rapidly oxidized to cystamine after application to the paper.

§ The methylated guanidines always gave two spots in this solvent.

‖ The dimethylureas were detectable only in high concentrations.

calculated from these fronts. The numbers in the table refer to those on the 'Derwent' colour chart (Reio, 1958) and Reio's conventions are used in Table 3. The colours could be described sufficiently well by comparing the spots directly with the chart, which was covered with a sheet of tracing paper, thus reducing the brilliance of its colours to about the same level as that of the spots on the paper.

Paper electrophoresis. Best results were obtained with Whatman no. 1 paper and 0·04 M-sodium barbiturate adjusted to pH 8·0. A potential gradient of 20 v/cm. was applied for an hour, and *N*-2:4-dinitrophenylethanolamine was used as an electroendosmosis marker. The amines were visualized after drying the paper strips in a current of air and spraying them with the reagents described above. The distances moved by some amines, corrected for electroendosmosis, are given in Table 4.

RESULTS

Preliminary experiments with single compounds dissolved in 1% sodium chloride showed that passage through the strongly basic resin removed all the amino acids except arginine, which passed through in 95% yield. Other classes of compounds that were retained were urinary pigments, proteins, pyrimidines, purines and all anions. Urinary pigments were impossible to remove completely from the resin on regeneration, although this did not have an adverse effect on its subsequent performance.

In the second step the pH of the supernatant of the stirred suspension of Zeo-Karb 226 stayed near neutrality and did not fall below 6·5 even at the end of the experiment. When chloride appeared in the effluent from the first column, owing to too low a flow-rate, the pH of the supernatant fell below 6·5, with a consequent possibility that some of the weaker bases were not adsorbed.

The recovery of the amines in all the stages up to and including the acid elution was found, in trial experiments, to be quantitative within experimental error, provided that the washing at each stage was adequate. Some losses occurred during the chromatographic separation for amines eluted

after methylamine (see Table 1). Also, amines with more than one basic group were not eluted from the resin under these conditions. Thus histamine, 1:4-diaminobutane and 1:5-diaminopentane gave no peaks before the pH 5 breakthrough, and satisfactory conditions for their elution have not yet been found.

Table 4. *Relative mobilities of some amines*

Electrophoresis was carried out on Whatman no. 1 paper strips with 0·04M-sodium barbiturate buffer, adjusted to pH 8·0, at 20v/cm. The distances are those moved by the amines in 1 hr., corrected for electroendosmosis.

Amine	Distance moved (cm.)
Adrenaline	3·5
n-Amylamine	7·0
*iso*Amylamine	6·8
Arginine	5·1
n-Butylamine	7·2
Creatinine	0·7
1:4-Diaminobutane	10·2
1:5-Diaminopentane	10·4
Diethanolamine	6·1
Diethylamine	7·9
Dimethylamine	11·7
Ethanolamine	8·5
Ethylamine	11·4
Glucosamine	2·5
Histamine	6·2
Hydroxylamine	1·4
5-Hydroxytryptamine	3·1
3-Hydroxytyramine	4·3
Methylamine	13·4
Nicotine	3·6
Noradrenaline	3·5
Phenethylamine	5·9
Piperidine	6·6
n-Propylamine	8·1
Pyrrolidine	9·4
Tryptamine	4·7
Tyramine	5·2

A useful feature of the method for desalting amines is that it worked not only with aliphatic and aromatic amines, but also with glutamic acid and arginine, which suggests that it is widely applicable to compounds containing amino groups.

The scope of these methods is indicated by Fig. 2, which shows elution curves (primary and secondary amines only) obtained from amine concentrates of 24 hr. urine samples. The first of these was obtained

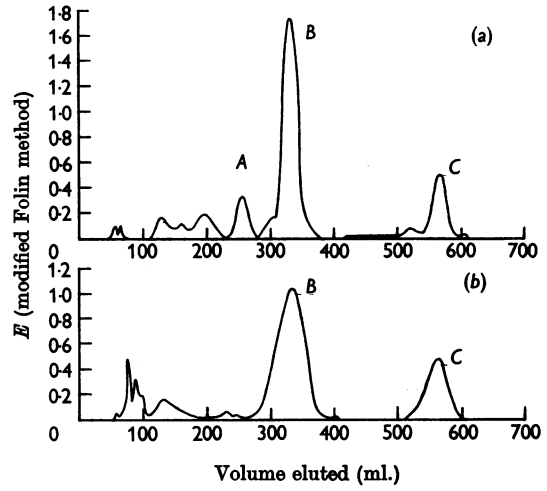

Fig. 2. Elution curves of the primary and secondary amines in concentrates corresponding to 500 ml. of urine applied to columns (2·0 cm. × 100 cm.) of Zeo-Karb 226 buffered at pH 7·3 and eluted with buffer at pH 5·0. (*a*), Curve obtained from urine (24 hr. volume, 1800 ml.) from a normal male on a normal diet; (*b*), curve from urine (24 hr. volume, 900 ml.) obtained from a normal male after a 5-day fast. *A*, Piperidine; *B*, dimethylamine; *C*, methylamine.

Table 5. *Excretion of piperidine, dimethylamine and methylamine*

The figures for each amine, as the free base, were calculated from elution curves obtained by the methods described in the text. The values for the normal diet are the average values from four different 24-hr. samples, with the range given underneath in parentheses. The values after fasting are from only one experiment.

Amine	Source	Excretion (mg./ml.)	Excretion (mg./24 hr.)	Reference
Piperidine	Normal human	3–20	.	Euler (1945)
	Normal male (normal diet)	3·8 (2·7–5·1)	5·7 (4·7–7·1)	Present work
	Normal male (fasting)	0	0	Present work
Dimethylamine	Normal human	5–40	.	Löffler (1935)
	Normal human	5	.	Dowden (1938)
	Normal male (normal diet)	10·9 (9·7–11·7)	17·0 (15·2–19·3)	Present work
	Normal male (fasting)	14·0	12·6	Present work
Methylamine	Normal male	82	.	Kapeller-Adler & Krael (1931)
	Normal female	100–150	.	Kapeller-Adler & Toda (1932)
	Normal male (normal diet)	3·3 (2·7–3·6)	5·0 (4·6–6·0)	Present work
	Normal male (fasting)	4·4	4·0	Present work

with the subject on a normal diet, the second on the fifth day of total fasting. The three major peaks were identified by the methods described as piperidine, dimethylamine and methylamine, all well established urinary constituents. Table 5 compares the excretion of these three amines with values from the literature.

The figures were calculated by measuring the areas under the peaks of the elution curve and correcting them for the total volumes of the 24 hr. specimens. Table 1 shows that glucosamine, methylamine and ethanolamine are not well resolved. It is therefore possible that the figures for the areas of the methylamine peaks may include small contributions from the other two amines, which occur in urine (Luck & Wilcox, 1953; Boas, 1956).

The most significant feature of these results is the absence of piperidine after fasting, which indicates that it is of dietary origin. It appears to be related structurally to lysine, from which it could arise by two alternative routes. The first is via the decarboxylation product, cadaverine, which was found, when fed to rabbits, to increase piperidine excretion, unlike lysine itself (Nordenström, 1951). The other pathway from lysine is that which Rothstein and his group (Rothstein & Miller, 1954; Rothstein & Greenberg, 1959) traced in the rat, via α-aminoadipic acid to glutaric acid. Pipecolic acid is an intermediate on this route, and on decarboxylation would also give piperidine.

Little is known about the origin of urinary amines, although they are generally regarded as arising from amino acids by decarboxylation. There is, however, a suggestion that methylamine and dimethylamine might be breakdown products of creatinine (Kapeller-Adler & Toda, 1932). The values of their excretion after fasting are not much lower than the normal ones, so that these two amines certainly appear to be derived from endogenous sources.

DISCUSSION

The methods developed in the present work were designed with the aim of being general for amines, and of minimizing the formation of alteration products. For this reason extreme conditions of temperature and of pH were avoided as much as possible. These considerations also governed the approach to the resolution of the amine concentrate. The chromatography of the dinitrophenyl derivatives (Asatoor & Dalgliesh, 1959) is limited to primary and secondary amines, and gas–liquid partition chromatography (James, Martin & Smith, 1952; James, 1952), although being potentially more rapid and convenient, is restricted to volatile amines. Weber (1951) used paper electrophoresis, but on the whole his results were disappointing. The use of ion-exchange chromatography provided a more general method, enabled the amines to be isolated as their hydrochlorides, and was capable of application on any scale.

SUMMARY

1. A method has been developed for treating biological fluids and extracts with ion-exchange resins to prepare a fraction consisting predominantly of the amines.

2. The resolution of this fraction has been obtained by the use of buffered columns of a weakly acidic ion-exchange resin.

3. Amines separated in this way have been isolated as their hydrochlorides by a chromatographic desalting technique, and identified by paper chromatography and paper electrophoresis.

4. The scope of these methods is illustrated with reference to the analysis of amines in urine, and figures are presented for the excretion of piperidine, dimethylamine and methylamine, both normally and after fasting.

I should like to thank Professor W. Robson for his encouragement and continued interest in this work.

REFERENCES

Asatoor, A. M. & Dalgliesh, C. E. (1959). *Biochem. J.* **73**, 26P.
Blau, K. & Robson, W. (1957). *Chem. & Ind.* p. 424.
Block, R. J., Durrum, E. L. & Zweig, G. (1955). *A Manual of Paper Chromatography and Paper Electrophoresis*, p. 225. New York: Academic Press Inc.
Boas, N. F. (1956). *Proc. Soc. exp. Biol., N.Y.*, **92**, 122.
Boyarkin, A. N. (1956). *Fiziologiya Rastenii*, **3**, 381. (*Chem. Abstr.* **50**, 16941 g.).
Bremner, J. M. & Kenten, R. H. (1951). *Biochem. J.* **49**, 651.
Dowden, H. C. (1938). *Biochem. J.* **32**, 455.
Euler, U. S. von (1945). *Acta pharm. tox., Kbh.*, **1**, 29.
Gardell, S. (1953). *Acta chem. scand.* **7**, 201.
Hamilton, P. B. (1958). *Analyt. Chem.* **30**, 914.
Herbst, E. J., Keister, D. L. & Weaver, R. H. (1958). *Arch. Biochem. Biophys.* **75**, 178.
Hirs, C. H. W., Moore, S. & Stein, W. H. (1953). *J. biol. Chem.* **200**, 493.
James, A. T. (1952). *Biochem. J.* **52**, 242.
James, A. T., Martin, A. J. P. & Smith, G. H. (1952). *Biochem. J.* **52**, 238.
Kapeller-Adler, R. & Krael, J. (1931). *Biochem. Z.* **235**, 394.
Kapeller-Adler, R. & Toda, K. (1932). *Biochem. Z.* **248**, 403.
Kirshner, N. & Goodall, McC. (1957). *J. biol. Chem.* **226**, 207.
Koch, F. C. & McMeekin, T. L. (1924). *J. Amer. chem. Soc.* **46**, 2066.
Lang, C. A. (1958). *Analyt. Chem.* **30**, 1692.
Löffler, H. (1935). *Hoppe-Seyl. Z.* **232**, 259.
Luck, J. M. & Wilcox, A. (1953). *J. biol. Chem.* **205**, 859.
McIlvaine, T. C. (1921). *J. biol. Chem.* **49**, 183.
Moffat, E. D. & Lytle, R. I. (1959). *Analyt. Chem.* **31**, 926.

Nordenström, B. E. W. (1951). *Acta pharm. tox., Kbh.*, **7**, 287.
Reio, L. (1958). *J. Chromat.* **1**, 338.
Rothstein, M. & Greenberg, D. M. (1959). *J. Amer. chem. Soc.* **81**, 4756.
Rothstein, M. & Miller, L. L. (1954). *J. biol. Chem.* **211**, 851.
Sanger, F. & Tuppy, H. (1951). *Biochem. J.* **49**, 463.
Schwyzer, R. (1952). *Acta chem. scand.* **6**, 219.
Smith, I. (1953). *Nature, Lond.*, **171**, 43.
Waley, S. G. & Watson, J. (1953). *J. chem. Soc.* p. 475.
Wall, J. S. (1953). *Analyt. Chem.* **25**, 950.
Weber, R. (1951). *Helv. chim. acta*, **34**, 2031.
Williams, R. J. (1951). Cited in Block, R J., Durrum, E. L. & Zweig, G. (1955). *A Manual of Paper Chromatography and Paper Electrophoresis*, p. 91. New York: Academic Press Inc.

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The Recovery of Injected Antigens from Rat Spleens

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(*Received* 27 *September* 1960)

The fate of heterologous proteins in experimental animals has been widely studied, particularly since the use of radioactive isotopes became widespread. The subject has been comprehensively reviewed by Haurowitz (1953, 1960) and Coons (1954). Protein-bound radioactivity has been shown to persist in the organs of rabbits injected with trace-labelled native proteins for up to 93 days (Crampton, Reller & Haurowitz, 1953). A similar persistence of radioactivity after injection of an extensively modified protein (anthranilylazo-ovalbumin) has also been demonstrated (Crampton, Reller & Haurowitz, 1952), despite the well-known differences between the immediate fate of native and extensively modified proteins in rabbits (Dixon, Bukantz & Dammin, 1951). Extensively modified azoproteins and iodoproteins leave the blood stream very rapidly (Haurowitz & Crampton, 1952; Francis & Hawkins, 1957), and during the first week after injection are found in much higher concentration in the tissues than are native proteins.

When the tissues in which antigen had been deposited were fractionated by differential centrifuging, Haurowitz & Crampton (1952) and Crampton *et al.* (1953) found that most of the antigen was associated with the mitochondrial fraction except during the first few minutes after injection. However, few studies have been reported of the chemical nature of the radioactivity retained in the tissues, so that little is definitely known about the persistence of the antigen itself. McMaster & Kruse (1951) have shown by a very sensitive immunological technique that unlabelled bovine γ-globulin injected into mice can be recovered from their livers in an immunologically reactive form 101 days later. Haurowitz & Walter (1955) injected [^{35}S]-sulphanilylazo bovine γ-globulin into rabbits, obtained protein from the liver 4 weeks later and hydrolysed it. Most of the ^{35}S was probably in the form of sulphanilylazotyrosine. This suggested that the determinant group of the protein was still intact. When Garvey & Campbell (1957) injected rabbits with [^{35}S]sulphanilylazo bovine serum albumin they recovered from the livers, up to 130 days later, labelled material which was highly active in sensitizing guinea pigs to the injected antigen. This material was associated with substances having an absorption maximum in the ultraviolet at 257·5 mμ. It is difficult to decide whether this material is likely to play a part in the development of the immune response, as the liver is probably not the site of antibody synthesis.

The work reported here was undertaken to try to provide more information about the chemical nature of the protein-bound radioactivity persisting in the spleen (since this organ is probably a site of antibody synthesis) after the injection of radioactive proteins. Two native heterologous proteins, trace-labelled with diazotized [^{35}S]sulphanilic acid, and one extensively modified protein, have been injected into rats. For comparison, trace-labelled rat serum albumin has also been injected. Determination of the amount of protein-bound ^{35}S precipitable from extracts made from the spleens of these rats by the appropriate rabbit antibody at various times after injection provided a measure of the persistence of free antigen. In view of Garvey & Campbell's findings, and also because of the well-known association of nucleic acids with protein synthesis, extraction and fractionation of the spleens were designed in such a way that extracts