A QUALITATIVE AND QUANTITATIVE STUDY OF THE SEPARATION OF UROPORPHYRIN OCTAMETHYL ESTERS I AND III BY DIOXAN CHROMATOGRAPHY

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Several methods are available for identification of uroporphyrin isomers; these include (a) dioxan chromatography (FALK AND BENSON[1]), (b) decarboxylation (EDMONDSON AND SCHWARTZ[2]) followed by lutidine chromatography of the coproporphyrin thus produced (ERIKSEN[3], RIMINGTON AND MILES[4]) or chromatography of the coproporphyrin esters (CHU, GREEN AND CHU[5]), (c) separation on Hyflo columns (CHU AND CHU[6]), (d) determination of melting point and mixed melting point (NICHOLAS AND RIMINGTON[7]), (e) infra-red spectrophotometry (FALK AND WILLIS[8]), (f) X-ray diffraction pattern (KENNARD AND RIMINGTON[9]). Some conflict between results obtained by dioxan chromatography and those obtained by other methods has been encountered both in this laboratory and by other authors (WATSON AND BERG[10], BOGORAD AND MARKS[11]). Some light has been thrown on the problem by the work of BOGORAD AND MARKS[11] who demonstrated, by means of labelled compounds, that when mixtures of uroporphyrin I and III esters were separated by dioxan chromatography the resulting spots each contained both isomers. Since the dioxan method is the least laborious there was a great need for its thorough investigation with a view to improving its reliability and sensitivity.

In the present investigation an attempt has been made to find optimal conditions for separating quantitatively mixtures of the isomers containing known amounts of uroporphyrin I varying between 0 and 100 % of the total ester. Estimation of the amount of ester in the I and the III positions respectively of the developed chromatogram was carried out by elution and spectrophotometry. It seems that quantitative separation of mixtures containing 50 % or less of the I isomer depends closely on the total amount of ester applied and subsequently eluted from the paper. In its final form, the technique may also be used for the separation of mixtures of unknown isomeric composition and for their quantitative evaluation.

MATERIALS

Solvents

The kerosene used was colourless commercial heating paraffin dried over calcium chloride and filtered. Chloroform, B.P., was washed three times with water, dried over calcium chloride and filtered. (It was found essential that washed chloroform, especially that used for elution, should not be used after five days storage.) Ethanolic

chloroform was prepared by adding ethanol to a concentration of 1 % (v/v) to chloroform treated as above. 1,4-Dioxan was purified by a method similar to that of EIGENBERGER[12] but with incorporation of a step designed to remove peroxides. Crude dioxan (1 l) was refluxed for 7 h (without the application of an air stream) in the presence of 120 ml of 4 % (w/v) hydrochloric acid. Peroxides were removed by shaking the mixture with excess of solid sodium sulphite: some solid potassium hydroxide was then added and the heavy white precipitate of inorganic salts removed by decantation and filtration. Shaking with potassium hydroxide pellets was then repeated until there was no further formation of an aqueous layer. The solvent was then dried over sodium wire and distilled as described by EIGENBERGER. Although free from peroxides, the dioxan at this stage still contained some aldehydic material which had not been removed by the original refluxing. This impurity was removed by fractional freezing, collecting the fraction which froze at 11.5°. The dioxan (b.p. 101–103°; m.p. 11.5°) was stored in dark bottles at 4°, at which temperature peroxide formation was reduced to a minimum.

Standard reference substances

Uroporphyrin I octamethyl ester, m.p. 293°, (uro I ester) was fraction Ai shown in Fig. 1 of the paper of RIMINGTON AND MILES[4]; further data concerning it are given by RIMINGTON AND SVEINSSON[13]. Uroporphyrin III octamethyl ester, m.p. 255–258°, (uro III ester) was the synthetic ester of TARLTON, MACDONALD AND BALTAZZI[14]. Coproporphyrin I tetramethyl ester, m.p. 253–256°, (copro I ester) was isolated from calf meconium. Coproporphyrin III tetramethyl ester, m.p. 155/170°, (copro III ester) was obtained from *Corynebacterium diphtheriae* by the method of GRAY AND HOLT[15]. Protoporphyrin IX dimethyl ester, m.p. 230°, was prepared from haemin by the method of GRINSTEIN[16]. Biosynthetic specimens were obtained by incubation of haemolysates of human erythrocytes with porphobilinogen, isolation of the uroporphyrin fraction and its esterification.

METHODS

Chromatography

Chromatography of the esters followed essentially the method of FALK AND BENSON[1]. Solutions of standard uro I and uro III esters were prepared at a concentration of 1 μg/10 μl in washed chloroform. This was achieved by weighing slightly more than the desired amount, dissolving, measuring the extinction at 405–406 mμ of a suitably diluted aliquot, using $E_{1\,cm}^{1\,\%} = 2300$ for uro III (RIMINGTON[17]) and $E_{1\,cm}^{1\,\%} = 2264$ for uro I (RIMINGTON[18]) and adjustment of volume. Mixtures of uro I and uro III esters were then prepared in varying ratios from these solutions, and stored at 4° when not in use. The concentrations of the chloroform solutions of the uro esters obtained from enzymic incubation were estimated by eqn. (1) below. Such solutions were then taken to dryness and the esters redissolved in sufficient chloroform to give 1 μg/10 μl.

Spots were applied by means of a micro-pipette, graduated in 5 μl, along a baseline 2 cm from one edge of a 21 cm square of Whatman No. 1 paper such that they would run with the grain of the paper.

Development of the chromatograms was carried out by the ascending method

at 22–26°. (Within this temperature range no variation in chromatographic behaviour occurred). Glass cylinders, one for each run (25.0 cm internal height; 13.2 cm internal diameter) were used. An inverted Petri dish was placed in position at the bottom of the tank and the solvent used to saturate the atmosphere was placed in the bottom of the tank, the lid greased carefully with yellow soft paraffin and placed in position. 15–30 min later the solvents for the developing mixture were pipetted into a second Petri dish, mixed well and the Petri dish placed on the inverted dish. The chromatogram, rolled in a cylinder, was then inserted and the lid replaced quickly and firmly. After development and drying, the spots were detected by their fluorescence in ultraviolet light. The tanks were cleaned, greased and equilibrated for each chromatogram.

First run: Atmosphere, 10 ml ethanolic chloroform; developing mixture, 4 ml kerosene + 6 ml ethanolic chloroform. Development was continued until the top of the spots as seen in ordinary light had moved 4 cm from the base-line (indicated by a mark made previously at the edge of the paper). Time 15–20 min. After drying the paper and marking lightly in pencil the positions of the spots (the dotted line in Figs. 1, 5 and 6) the base of the paper was cut off 0.5 cm below the bottom of the spots. No isomer separation takes place during this run, which is designed merely to separate the esters from impurities which are left on the base-line.

Second run: Atmosphere, 7 ml dioxan; developing mixture, 4.0 ml kerosene + 1.0 ml dioxan (effect of variation in volume of dioxan was tested, see RESULTS, but unless otherwise indicated a volume of 1.0 ml was used). Development was continued until the solvent had reached the top of the paper (1.5 h). The paper was dried thoroughly (30 min in a warm place) and the spots marked very lightly in pencil.

For calculation of R_F values, the base-line was taken as the centre of the spot after the first run.

Elution of chromatograms

Fluorescent spots were marked lightly with pencil, cut out from the paper and each eluted in 3 ml of freshly washed chloroform by standing in stoppered tubes for 1 h with occasional shaking. The extinction of the chloroform solutions was measured in a Unicam model SP. 500 photoelectric spectrophotometer, at a 1 cm light path. The complete process, from application of material to spectrophotometric reading, was carried out within 4 h. The ester content of the eluates was calculated from the following expression (for derivation, see APPENDIX):

$$\mu g \text{ uro ester} = [2D_{405-6} - (D_{380} + D_{430})] \times 2.721 \times V \qquad (1)$$

where V = volume of solution;
D = extinction at wave-length indicated by the subscript.

Decarboxylation

5–20 μg of the uro esters obtained by enzymic incubation were decarboxylated by the method of EDMONDSON AND SCHWARTZ[2]. The decarboxylation tubes were sealed at a pressure equal to 1.5 mm Hg after holding them at that pressure for 10 min. Decarboxylation of this quantity of uro ester at this pressure yielded 100% recovery of the decarboxylation product, coproporphyrin. If the tubes were sealed in air or while attached to the water pump, however, very large or complete destruction

of the porphyrin ensued, an effect also noted by EDMONDSON AND SCHWARTZ[2]. The free coproporphyrin thus produced was prepared for lutidine chromatography by one of the following means: (a) pH was adjusted to 4 with saturated sodium acetate and the coproporphyrin transferred quantitatively to ether; the ether was washed three times with water and the aqueous washings extracted with fresh ether. The ethereal solutions were combined, washed with a very small quantity of water and evaporated to dryness. (b) As (a) followed by esterification and subsequent hydrolysis by the method of FALK, DRESEL, BENSON AND KNIGHT[19] using methanol–H_2SO_4 (95:5, v/v) as the esterification mixture.

Samples prepared by either (a) or (b) were dissolved in 2 *N* NH_4OH for lutidine chromatography. When duplicate samples were taken and one prepared by method (a) the other by (b) they were found to give the same result on lutidine chromatography and, therefore, method (a) was made the standard method of preparation because of its relative simplicity.

Lutidine chromatography

Isomer identification of the free coproporphyrin obtained by decarboxylation of the uro esters was carried out essentially by the method of ERIKSEN[3].

RESULTS

Variation in ratio of kerosene to dioxan

A series of runs was made to determine the optimal ratio of kerosene to dioxan required to separate quantitatively 1 μg of a 50:50 mixture of uro I and III esters. The results set out in Table I demonstrate clearly that decrease in the concentration of dioxan caused less of the I isomer to be carried up with the III isomer. It was apparent that the optimal ratio required to separate 1 μg of a 50:50 mixture was of the order of 4.0:1.0.

TABLE I

EFFECT OF VARIATION IN RATIO OF KEROSENE TO DIOXAN ON SEPARATION OF 1 μg OF A 50:50 MIXTURE OF URO I AND III ESTERS

Kerosene: dioxan	*Ester eluted*		
	I position (μg)	*III position (μg)*	*Apparent ratio I:III*
4.0:1.30	0.27	0.49	36:64
4.0:1.25	0.24	0.50	32:68
4.0:1.20	0.28	0.52	35:65
4.0:1.15	0.32	0.47	41:59
4.0:1.10	0.26	0.43	38:62
4.0:1.05	0.52	0.47	53:47
4.0:1.00	0.51	0.45	53:47

Variation in quantity of ester mixture applied

The separation was studied of a range of quantities of a 50:50 mixture of uro I and III esters by means of kerosene–dioxan mixtures of ratio 4.0:1.00 and 4.0:1.05 respectively. On visual examination, spots could be seen in the I and III positions in all

cases, but it was obvious that along the series the uro I spot increased in intensity and size much more than did the III spot, (Fig. 1). The results of elution from two chromatograms, set out in Table II, demonstrate that the quantity of ester eluted from the I position became an increasing proportion of the total ester applied as the amount

TABLE II

EFFECT ON SEPARATION OF VARIATION IN AMOUNT OF 50:50 MIXTURE OF URO I AND III ESTERS APPLIED

Kerosene: dioxan	*Ester applied (μg)*	*Ester eluted*		
		I position (μg)	*III position (μg)*	*Apparent ratio I:III*
4.0:1.05	0.30	0.02	0.18	10:90
	0.50	0.17	0.28	38:62
	0.75	0.27	0.39	41:59
	1.00	0.52	0.47	52:48
	1.50	0.95	0.70	58:42
	2.00	1.34	0.72	65:35
4.0:1.00	0.30	0.03	0.17	15:85
	0.50	0.16	0.28	36:64
	0.75	0.36	0.41	47:53
	1.00	0.51	0.45	53:47
	1.50	0.92	0.61	60:40
	2.00	1.37	0.71	66:34

of the latter increased; the theoretical 50 % separation was obtainable only at one point. The two solvent ratios used gave essentially similar results. In subsequent work a kerosene–dioxan ratio of 4.0:1.0 was employed.

Recovery of esters by elution from chromatograms

From a comprehensive survey of our data, elution from chromatograms was found to vary between the extreme limits of 50 % and 100 %, most usually approximating

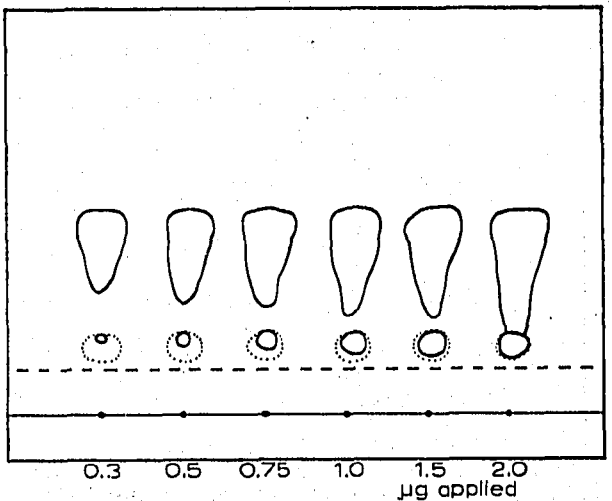

Fig. 1. Paper chromatogram obtained by applying a series of amounts of a 50:50 mixture of uroporphyrin I and III esters (kerosene–dioxan = 4.0:1.0). Dotted lines mark positions of spots after the first run and the solid lines their positions after final development. The bottom of the paper was cut off along the horizontal dashed line after the first run.

70%. The reason for this variability remains unknown; it does not depend upon greater stability or preferential elution of one or other isomer since a similar range of variability was found at all isomer ratios.

It was noted that better agreement with expected values was obtained in any

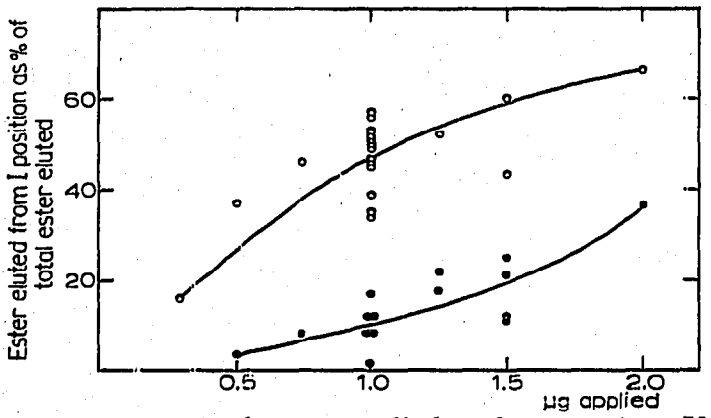

Fig. 2. Relationship between amount of ester applied and separation. Values were obtained by applying a series of amounts of two different ratios of uroporphyrin I and III esters to several chromatograms each run in kerosene–dioxan = 4.0:1.0. O Uro I ester : uro III ester = 50:50; ● uro I ester:uro III ester = 25:75.

separation by basing calculations upon the total ester eluted (I + III positions) rather than upon the total applied to the paper. The importance of this observation will become apparent in the next section.

Variation in ratio of uro I and III esters applied

Runs were made on a range of mixtures of uro I and III esters using a kerosene–dioxan ratio of 4.0:1.0. Each mixture was applied over a range of quantities and the spots in the I and III position were eluted and measured in the usual way. Fig. 2 relates the apparent isomer distribution to the amount of known mixture applied in the case of two mixtures of standards (I:III = 25:75 and I:III = 50:50 respectively). Whilst there is some evidence of interdependence, this is unimpressive. When, however, the proportion of apparent uro I was plotted against the total ester

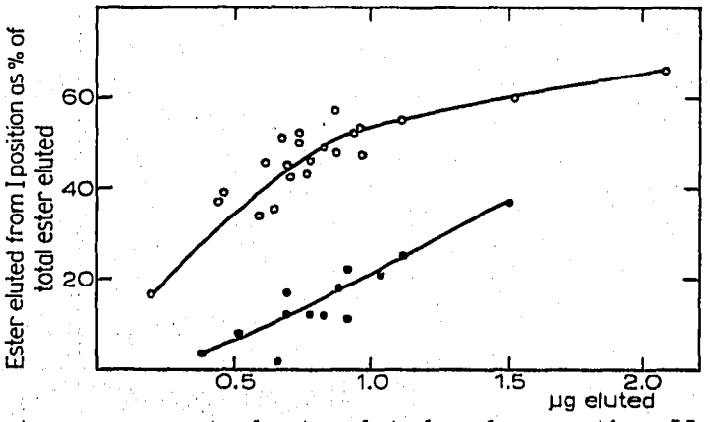

Fig. 3. Relationship between amount of ester eluted and separation. Values were obtained from the same chromatograms as those for Fig. 2. "µg eluted" refers to the sum of the amounts eluted from the I and III positions. O Uro I ester : uro III ester = 50:50; ● uro I ester : uro III ester = 25:75.

eluted from the paper, as in Fig. 3, a clearly defined relationship was discernible, for each of these two mixtures. When comparison was made of a range of ratios of uro I and III esters, it became apparent that with increase in the proportion of the I isomer in the mixture applied quantitative separation was increasingly less dependent on the amount of ester eluted.

As there exists a different optimal amount to be eluted for each ratio of I to III,

TABLE III

RELATIONSHIP OF A, B AND C IN EXPRESSION (2) TO THE TOTAL μg OF ESTER ELUTED

x = total μg of ester (I + III) eluted,
$A = 30.77 - 10.51x - 21.49x^2 + 16.04x^3$
$B = 0.7509 - 0.3755x + 0.5997x^2 - 0.5619x^3$
$C = -0.000999 + 0.005780x - 0.004834x^2 + 0.004361x^3$

x*	A	B	C
0.40	24.2	0.661	0.00082
0.42	23.8	0.657	0.00090
0.44	23.4	0.654	0.00098
0.46	23.0	0.650	0.00106
0.48	22.6	0.647	0.00114
0.50	22.2	0.643	0.00123
0.52	21.8	0.639	0.00131
0.54	21.4	0.635	0.00140
0.56	21.0	0.630	0.00149
0.58	20.6	0.625	0.00158
0.60	20.2	0.620	0.00167
0.62	19.8	0.615	0.00177
0.64	19.5	0.609	0.00186
0.66	19.1	0.603	0.00197
0.68	18.7	0.596	0.00207
0.70	18.4	0.589	0.00218
0.72	18.1	0.582	0.00229
0.74	17.7	0.574	0.00240
0.76	17.4	0.565	0.00252
0.78	17.1	0.556	0.00264
0.80	16.8	0.547	0.00277
0.82	16.6	0.536	0.00290
0.84	16.3	0.526	0.00303
0.86	16.1	0.514	0.00317
0.88	15.8	0.502	0.00332
0.90	15.6	0.489	0.00347
0.92	15.4	0.475	0.00362
0.94	15.2	0.461	0.00379
0.96	15.1	0.446	0.00395
0.98	14.9	0.430	0.00413
1.00	14.8	0.413	0.00431
1.02	14.7	0.396	0.00450
1.04	14.6	0.377	0.00469
1.06	14.6	0.357	0.00489
1.08	14.6	0.337	0.00510
1.10	14.6	0.316	0.00532
1.12	14.6	0.293	0.00554
1.14	14.6	0.270	0.00577
1.16	14.7	0.245	0.00601
1.18	14.8	0.220	0.00626
1.20	14.9	0.193	0.00651

* Linear interpolation may be used for intermediate values of x, but the table should not be extended beyond 0.40 and 1.20.

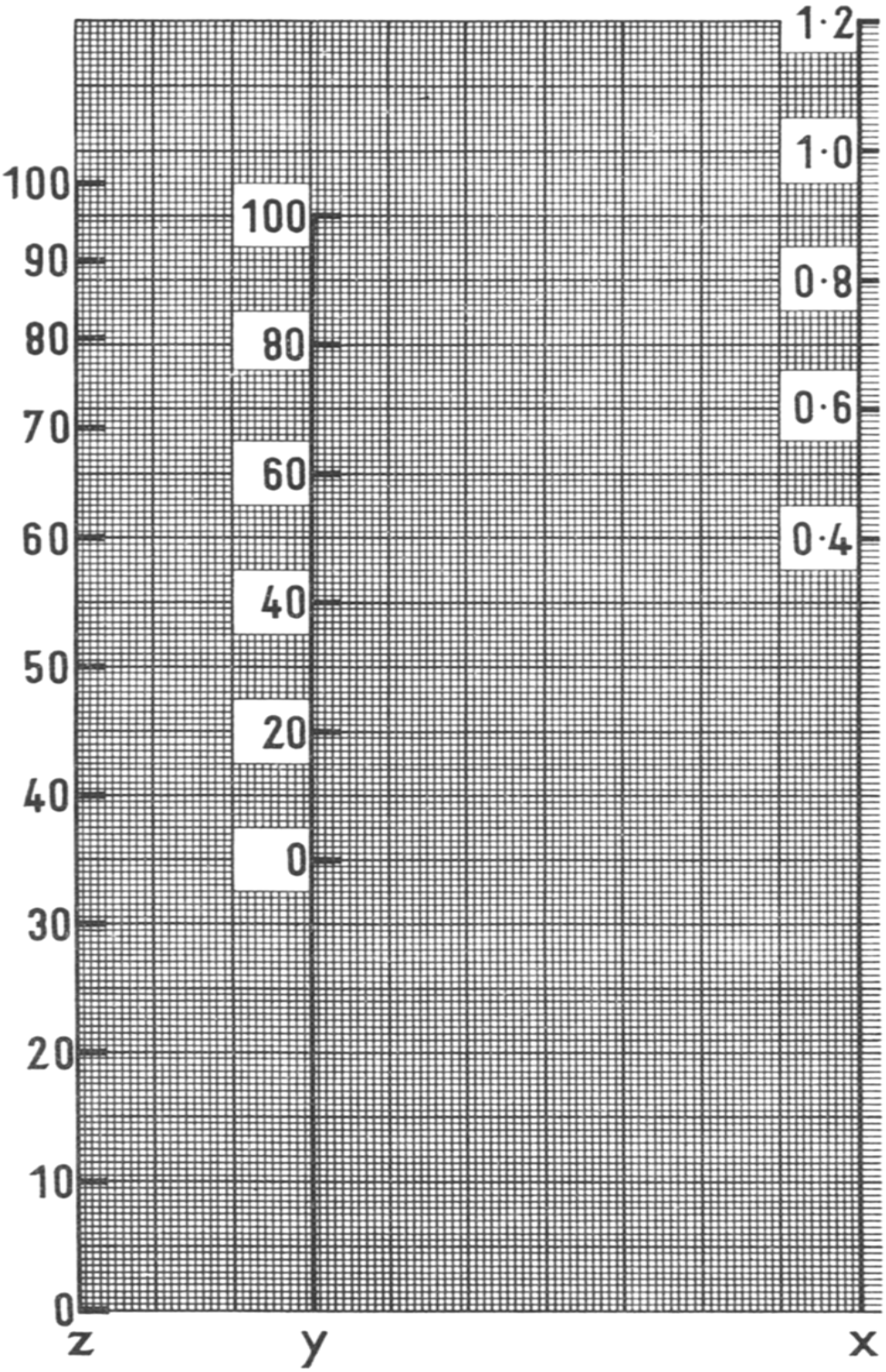

Fig. 4. Nomograph for correction of ratios of uro isomers found by elution. Connect "μg of total uro ester (I + III) eluted" (scale x) with "apparent percentage of uro I isomer found by elution" (scale y) and read off "true percentage of I isomer" (scale z).

it is necessary to correct the ratio found against the amount eluted. This may be done by use of the expression:

$$z = A + By + Cy^2 \qquad (2)$$

where z = the true percentage of the I isomer;
y = the apparent percentage of the I isomer found by elution,
and A, B and C are constants related to the total μg of ester eluted (x) and which may be found by reference to Table III.

For derivation of expression (2) see APPENDIX. Alternatively, correction may be made by use of the nomograph illustrated in Fig. 4, where x, y and z have the same meaning as in eqn. (2) and Table III.

Table IV sets out the results from several chromatograms of the separation of a range of isomer ratios; both the elution figures and the corrected values are included. It can be seen that when between 17.5 and 100% of uro I is present in a mixture of I and III, it may be detected and estimated within an accuracy of 7% (mean error = 2.2%) by use of eqn. (2). The nomograph presents a more simple method of correction but its efficacy falls off when between 90% and 100% of the I isomer is found by elution. Since when $y = 0$, $z = A$ in eqn. (2), erroneous results would be obtained in attempts to correct a zero value. In practice, therefore, if no spot is visible in the I position it may simply be concluded that between 0 and 17.5% of uro I is present. Quantities less than 17.5% cannot be detected by elution, but 12.5–17.5% of the I isomer may be detected visually by the tailing of the spot in the III position onto, but not into, the I position.

When a sample of chromatography paper other than Whatman No. 1, namely "Ederol No. 202", was tested, variation in the pattern of separation occurred such that mathematical correction did not yield a theoretical result. It seems, therefore, that the relationship governed by eqn. (2) breaks down with change of paper.

Fig. 5 has been prepared from a typical chromatogram run on 1 μg each of ratios of uro I and III esters ranging between 0:100 and 100:0.

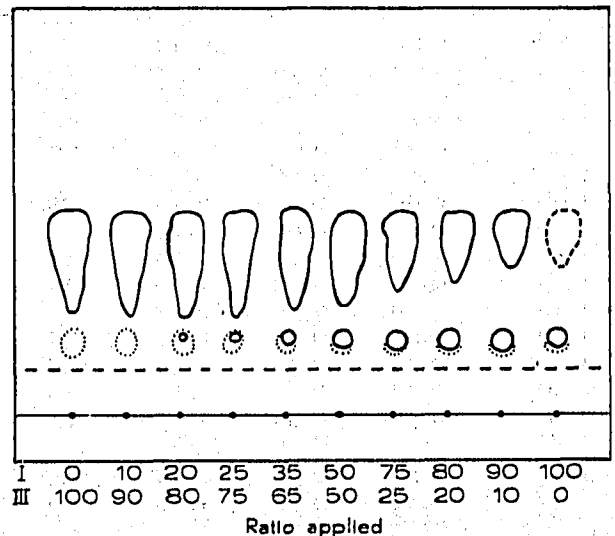

Fig. 5. Paper chromatogram of a series of ratios of uroporphyrin I and III esters. 1.0 μg of each ratio was applied (kerosene–dioxan = 4.0:1.0). Dotted lines mark positions of spots after the first run, solid lines their positions after final development and dashed line the position of a very weak spot after final development. The bottom of the paper was cut off along the horizontal dashed line after the first run.

TABLE IV

SEPARATION OF VARYING AMOUNTS OF A RANGE OF RATIOS OF URO I AND III ESTERS IN KEROSENE–DIOXAN (4.0 : 1.0)

Ester mixture applied				*Ester eluted*					*Apparent ratio (I : III) corrected*	
Ratio I : III	*I (μg)*	*III (μg)*	*I + III (μg)*	*I position (μg)*	*III position (μg)*	*Sum of I + III positions (μg)*	*Recovery (%)*	*Apparent ratio I : III*	*By nomograph*	*By eqn. (2)*
0:100	0.00	1.00	1.00	0.00	0.55	0.55	55	0:100		
0:100	0.00	1.00	1.00	0.00	0.62	0.62	62	0:100		
0:100	0.00	1.00	1.00	0.00	0.66	0.66	66	0:100		
0:100	0.00	1.00	1.00	0.00	0.67	0.67	67	0:100		
0:100	0.00	1.00	1.00	0.00	0.68	0.68	68	0:100		
0:100	0.00	1.00	1.00	0.00	0.69	0.69	69	0:100		
0:100	0.00	1.00	1.00	0.00	0.70	0.70	70	0:100		
0:100	0.00	1.00	1.00	0.00	0.79	0.79	79	0:100		
0:100	0.00	1.25	1.25	0.00	0.97	0.97	77	0:100		
0:100	0.00	1.50	1.50	0.00	1.04	1.04	69	0:100		
0:100	0.00	1.50	1.50	0.00	1.08	1.08	72	0:100		
0:100	0.00	2.00	2.00	0.14	1.32	1.46	73	10:90		
0:100	0.00	2.00	2.00	0.51	0.96	1.47	73	35:65		
10:90	0.10	0.90	1.00	0.00	0.53	0.53	53	0:100		
10:90	0.10	0.90	1.00	0.00	0.69	0.69	69	0:100		
10:90	0.15	1.35	1.50	0.00	0.73	0.73	49	0:100		
10:90	0.10	0.90	1.00	0.00	0.77	0.77	77	0:100		
10:90	0.15	1.35	1.50	0.00	1.02	1.02	68	0:100		
10:90	0.20	1.80	2.00	0.57	0.97	1.54	77	37:63		
17:83	0.175	0.825	1.00	0.03	0.66	0.69	69	5:95	22:78	22:78
20:80	0.20	0.80	1.00	0.03	0.71	0.74	74	4:96	20:80	20:80
20:80	0.20	0.80	1.00	0.05	0.71	0.76	76	6:94	21:79	21:79
20:80	0.30	1.20	1.50	0.04	0.79	0.83	55	5:95	19:81	19:81
20:80	0.25	1.00	1.25	0.09	0.82	0.91	73	10:90	20:80	21:79
25:75	0.125	0.375	0.50	0.02	0.37	0.39	78	5:95	27:73	27:73
25:75	0.188	0.563	0.75	0.04	0.49	0.53	70	9:91	27:73	26:74
25:75	0.25	0.75	1.00	0.02	0.64	0.66	66	3:97	20:80	20:80
25:75	0.25	0.75	1.00	0.12	0.57	0.69	69	17:83	30:70	29:71
25:75	0.25	0.75	1.00	0.08	0.61	0.69	69	12:88	26:74	26:74
25:75	0.25	0.75	1.00	0.06	0.66	0.72	72	8:92	23:77	23:77
25:75	0.25	0.75	1.00	0.06	0.67	0.73	73	8:92	23:77	23:77
25:75	0.25	0.75	1.00	0.10	0.67	0.77	77	13:87	25:75	24:76
25:75	0.375	1.125	1.50	1.10	0.73	0.83	55	12:88	24:76	23:77
25:75	0.313	0.938	1.25	0.16	0.73	0.89	71	18:82	26:74	26:74
25:75	0.313	0.938	1.25	0.20	0.72	0.92	74	22:78	29:71	28:72
25:75	0.375	1.125	1.50	0.11	0.82	0.93	61	12:88	21:79	21:79
25:75	0.375	1.125	1.50	0.22	0.82	1.04	69	21:79	25:75	25:75
25:75	0.375	1.125	1.50	0.28	0.84	1.12	75	25:75	26:74	25:75
25:75	0.50	1.50	2.00	0.56	0.95	1.51	75	37:63	Beyond limits	
30:70	0.30	0.70	1.00	0.10	0.53	0.63	63	16:84	31:69	30:70
30:70	0.375	0.875	1.25	0.22	0.78	1.00	80	22:78	27:73	26:74
35:65	0.35	0.65	1.00	0.15	0.54	0.69	69	22:78	34:66	33:67
35:65	0.438	0.813	1.25	0.28	0.66	0.94	75	30:70	34:66	33:67

(continued on p. 151)

TABLE IV (*continued*)

Ester mixture applied				*Ester eluted*					*Apparent ratio (I: III) corrected*	
Ratio I: III	*I (μg)*	*III (μg)*	*I + III (μg)*	*I position (μg)*	*III position (μg)*	*Sum of I + III positions (μg)*	*Recovery (%)*	*Apparent ratio I: III*	*By nomograph*	*By eqn. (2)*
40:60	0.40	0.60	1.00	0.21	0.51	0.72	72	29:71	38:62	37:63
40:60	0.50	0.75	1.25	0.32	0.66	0.98	73	33:67	35:65	33:67
50:50	0.15	0.15	0.30	0.03	0.17	0.20	67	15:85	Beyond limits	
50:50	0.25	0.25	0.50	0.16	0.28	0.44	88	36:64	50:50	49:51
50:50	0.50	0.50	1.00	0.18	0.28	0.46	46	39:61	51:49	50:50
50:50	0.50	0.50	1.00	0.20	0.39	0.59	59	34:66	45:55	43:57
50:50	0.50	0.50	1.00	0.28	0.33	0.61	61	46:54	53:47	52:48
50:50	0.50	0.50	1.00	0.22	0.42	0.64	64	34:66	44:56	43:57
50:50	0.50	0.50	1.00	0.35	0.32	0.67	67	52:48	55:45	55:45
50:50	0.50	0.50	1.00	0.31	0.37	0.68	68	46:54	50:50	50:50
50:50	0.75	0.75	1.50	0.30	0.40	0.70	70	43:57	49:51	48:52
50:50	0.50	0.50	1.00	0.38	0.35	0.73	73	52:48	54:46	54:46
50:50	0.50	0.50	1.00	0.37	0.37	0.74	74	50:50	53:47	52:48
50:50	0.75	0.75	1.50	0.33	0.43	0.76	51	43:57	47:53	46:54
50:50	0.375	0.375	0.75	0.35	0.42	0.77	102	45:55	49:51	49:51
50:50	0.50	0.50	1.00	0.41	0.42	0.83	83	49:51	50:50	50:50
50:50	0.50	0.50	1.00	0.50	0.37	0.87	87	57:43	55:45	55:45
50:50	0.625	0.625	1.25	0.49	0.45	0.94	75	52:48	50:50	50:50
50:50	0.50	0.50	1.00	0.51	0.45	0.96	96	53:47	50:50	50:50
50:50	0.50	0.50	1.00	0.46	0.52	0.98	98	47:53	45:55	45:55
50:50	0.50	0.50	1.00	0.62	0.49	1.11	111	56:44	49:51	49:51
50:50	0.75	0.75	1.50	0.92	0.61	1.53	102	60:40	Beyond limits	
50:50	1.00	1.00	2.00	1.37	0.71	2.08	104	66:34	Beyond limits	
75:25	0.75	0.25	1.00	0.48	0.17	0.65	65	74:26	74:26	75:25
75:25	0.75	0.25	1.00	0.57	0.19	0.76	76	75:25	71:29	73:27
75:25	0.75	0.25	1.00	0.65	0.19	0.84	84	77:23	80:20	76:24
75:25	1.125	0.375	1.50	0.76	0.14	0.90	60	84:16	78:22	81:19
75:25	1.125	0.375	1.50	0.97	0.23	1.20	80	81:19	75:25	73:27
80:20	0.80	0.20	1.00	0.45	0.12	0.57	57	79:21	81:19	79:21
80:20	0.80	0.20	1.00	0.47	0.12	0.59	59	80:20	81:19	80:20
80:20	0.80	0.20	1.00	0.59	0.15	0.74	74	80:20	78:22	79:21
80:20	1.20	0.30	1.50	0.71	0.17	0.88	59	81:19	73:27	77:23
80:20	1.20	0.30	1.50	0.85	0.11	0.96	64	89:11	80:20	86:14
90:10	0.90	0.10	1.00	0.60	0.07	0.67	67	90:10	90:10	88:12
90:10	0.90	0.10	1.00	0.66	0.04	0.70	70	94:6	95:5	93:7
90:10	0.90	0.10	1.00	0.65	0.06	0.71	71	92:8	92:8	91:9
90:10	1.35	0.15	1.50	0.91	0.04	0.94	63	97:3	90:10	95:5
90:10	1.35	0.15	1.50	0.92	0.11	1.03	69	89:11	80:20	86:14
100:0	1.00	0.00	1.00	0.60	0.00	0.60	60	100:0	108:0	99:1
100:0	1.00	0.00	1.00	0.71	0.00	0.71	71	100:0	103:0	99:1
100:0	1.00	0.00	1.00	0.74	0.00	0.74	74	100:0	102:0	99:1
100:0	1.00	0.00	1.00	0.77	0.00	0.77	77	100:0	100:0	99:1
100:0	1.00	0.00	1.00	0.82	0.00	0.82	82	100:0	98:2	99:1
100:0	1.25	0.00	1.25	1.04	0.00	1.04	83	100:0	90:10	99:1
100:0	1.50	0.00	1.50	1.18	0.00	1.18	79	100:0	88:12	99:1
100:0	2.00	0.00	2.00	1.62	0.00	1.62	81	100:0	Beyond	limits

TABLE V

R_F VALUES OF ESTERS OF PORPHYRINS WITH 2–8 CARBOXYL GROUPS RUN IN KEROSENE–DIOXAN (4.0:1.0)

Ester	*Source*	R_F
Uroporphyrin I	Standard reference	0.00
Coproporphyrin I	Standard reference	0.04
Uroporphyrin III	Standard reference	0.23
Coproporphyrin III	Standard reference	0.66
Protoporphyrin IX	Standard reference	0.74
Uroporphyrin I	Enzymic	0.00
Uroporphyrin III	Enzymic	0.21
Heptacarboxylic porphyrin III?	Enzymic	0.40
Hexacarboxylic porphyrin III?	Enzymic	0.50
Pentacarboxylic porphyrin III?	Enzymic	0.58
Coproporphyrin III	Enzymic	0.66

Separation of esters of porphyrins with 2–8 carboxyl groups

Table V sets out the R_F values of esters of uroporphyrin, coproporphyrin and protoporphyrin run in a kerosene–dioxan mixture of 4.0:1.0. In addition, the table shows R_F values of three spots found between uro III and copro III positions when the uro fraction obtained from enzymic incubation of porphobilinogen is chromatographed (see Fig. 6). These three spots are assumed to be the esters of porphyrins belonging to the III series with seven, six and five carboxyl groups per molecule respectively (see DISCUSSION).

DISCUSSION

When dioxan from which peroxides had not been removed was used, very poor elution yields of the esters were obtained and separation was far from quantitative; hence

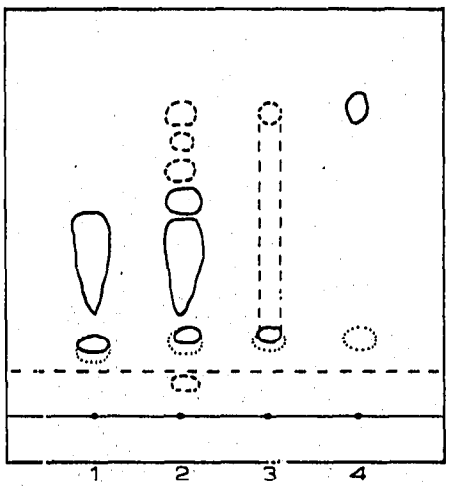

Fig. 6. Paper chromatogram relating chromatographic behaviour of uroporphyrin ester obtained enzymically to standard porphyrin esters (kerosene–dioxan = 4.0:1.0). (1) 50:50 mixture of uroporphyrin I and III esters; (2) material obtained by separation and esterification of uroporphyrin fraction after enzymic incubation of porphobilinogen; (3) coproporphyrin I ester; (4) coproporphyrin III ester. Dotted lines mark positions of spots after the first run, the solid lines their positions after final development and the dashed lines the positions of weak spots after final development. The bottom of the paper was cut off along the horizontal dashed line after the first run.

the need for peroxide-free dioxan was established. FALK AND BENSON[1] used a kerosene–dioxan ratio of 4.0:1.5 but pointed out that the optimal ratio required for separation of mixtures of uroporphyrin I and III esters varied with different batches of dioxan. Thus, in the work reported here the optimal ratio found (4.0:1.0) was relatively low with respect to dioxan as compared with the FALK AND BENSON ratio, obviously as a result of the higher purification of the dioxan.

It seems that the amount of uro I found in the I position after running a chromatogram depends on several factors, including:

(a) the amount of uro I applied,
(b) its partial loss during chromatography,
(c) the amount of uro III applied with it.

In the same way, the amount of uro III present in the III position depends on:

(a) the amount of uro III applied,
(b) its partial loss during chromatography,
(c) the amount of uro I applied with it.

There can be no doubt that some form of molecular association occurs between the two isomers (NICHOLAS AND RIMINGTON[7], EDMONDSON AND SCHWARTZ[2] and BOGORAD AND MARKS[11]). This is further revealed by the present investigation for whereas all the uro I applied alone remains in the I position, yet the presence of uro III will cause some of the I to move up to the III position. Similarly, the addition of uro I to uro III will cause some III to stay down. Contribution by each of the above factors results in a very delicate equilibrium being established which governs the separation of a given mixture of the isomers.

The complexity of the equilibrium is further demonstrated by the following observation: under conditions where a 50:50 mixture separated such that equal amounts were eluted from the I and III positions, several applications of such mixture were run and the ester eluted from all the I positions combined and that from all the III positions combined. When these were each re-chromatographed and estimated under conditions identical with those above, corrected separations of 65:35 (I:III) and 35:65 (I:III) respectively were noted.

Since the way in which a given ratio separates depends not only on that ratio but also on the amount of ester present on the paper, it follows that there is an optimal amount of ester which should be present so that the correct ratio will be found on elution. As the optimal amount was found, however, to vary with the ratio, for example 0.8 μg for a 50:50 mixture and 1.1 μg for a 25:75 (I:III) mixture, inevitable "under-" or "over-loading" in practice necessitates a correction of the ratio found on elution in order to arrive at the true ratio.

Our data have shown, moreover, that the "optimal amount" relates to the total quantity of ester elutable from the paper rather than to the amount applied. Recoveries by elution varied over a range of 50–100 %, most generally being of the order of 70 %: it was noted, however, that variation in recovery of a given amount of ester was reflected in the separation and that such consequent irregularities in separation could be corrected by a formula which contained a term for the amount eluted. It is, therefore, assumed that low recovery results from a loss of ester early in the chromatographic process, perhaps during the first run, before separation has taken place, otherwise such a relationship between separation and amount eluted would not be apparent. 0.5 μg of a 50:50 mixture chromatographed with 88 %

recovery gave essentially the same result as 1.0 μg chromatographed with 46% recovery. That the relationship between separation and amount eluted is not due to a greater relative stability of one isomer is shown by the fact that the same range of yield was found for all ratios of I to III.

No explanation of the physicochemistry of the chromatographic behaviour of the mixed isomers can be offered, but even without such understanding it is considered that with the aid of the empirically-derived formula (2), which relates the equilibrium set up on the paper by a given amount of mixed isomers to their true ratio, the ratio of uro I to uro III in a given mixture may be determined with 7% accuracy.

The formula was found to give accurate results for all ratios of I to III when between 0.4 and 1.2 μg of total uro ester was eluted. Since recovery lay between 50 and 100%, then 1.0 μg of any mixture of uro esters is the most suitable amount to be applied. Uro esters derived from biological sources or from biochemical reactions often contain porphyrin esters other than uro and their proportion had to be determined roughly by a preliminary dioxan chromatogram before an amount of the sample was known which contained approximately 1 μg of uro itself: for example, if the preliminary chromatogram showed approximately 30% of fluorescent material other than uro then 1.5 μg of the sample was applied.

The esters other than uro obtained from enzymic incubation and present in the uro fraction are seen on chromatograms as one to four spots above the uro III position. The most mobile coincides with standard copro III and is only present in very faint traces, if at all, when copro has been separated from uro prior to chromatography. The three spots lying between uro III and copro III are assumed to be the esters of porphyrins of the III series with seven, six and five carboxyl groups per molecule respectively (see Table V and Fig. 6). That they do belong to the III series is deduced from the fact that under these chromatographic conditions uro I and copro I have R_F values of 0.00 and 0.02 respectively and it seems most likely that any esters of the I series with a number of carboxyl groups intermediate between eight and four would also have very low R_F values. The materials being considered have R_F values, however, 0.40, 0.50 and 0.58 respectively. That they contain seven, six and five carboxyl groups per molecule, respectively, is deduced from their positions between uro III and copro III and relative to each other. Those spots provisionally ascribed to penta- and hexacarboxylic porphyrin esters were always very faint, but the spot assumed to contain heptacarboxylic porphyrin ester was often of very significant concentration. The porphyrin responsible for this spot has been designated pseudouroporphyrin by FALK[20], FALK, DRESEL, BENSON AND KNIGHT[19] and FALK AND DRESEL[21]. This spot was eluted at the same time as the uro ester spots and its concentration, as a percentage of the uro, determined with only a small error by using the same $E^{1\%}_{1cm}$ and k values as for uro, (eqn. (1)). It was the intensity of this spot which needed to be allowed for in assessing the amount of sample required to be applied such that the spot would contain approximately 1 μg of uro. It was, of course, always necessary when working with unknowns to run standard mixtures on the same chromatogram and to ascertain that they gave theoretical ratio values on elution and corrected calculation. 100% uro I ester, 100% uro III ester and a 50:50 mixture of these were routinely used in the present investigation.

The method has been used, and found to yield highly reproducible results, in separating the I and III isomers of uro derived from enzymic incubations of por-

phobilinogen. When the uro samples contained a mixture of uro I and III esters, but very little or no pseudouroporphyrin, results by dioxan chromatography were found to agree very closely with those obtained by chemical decarboxylation followed by lutidine chromatography of the copro formed. (The ratio of copro I to copro III after development of lutidine chromatograms was assessed by visual examination of their fluorescence in ultraviolet light. The two spots, being of similar size and shape, may be compared with respect to their relative intensity.) When the pseudouroporphyrin concentration was high, it was found that the ratio of uro I to (uro III + pseudouroporphyrin) agreed closely with results by decarboxylation and lutidine chromatography. This can be taken as further evidence that the spot found above uro III and known as pseudouroporphyrin is a compound which decarboxylates to yield copro III.

After the first run (kerosene–chloroform) of samples derived from enzymic incubation, two spots appear on the paper. The faster-moving spot gives rise after the second run to the characteristic spots of uro I, uro III, and pseudouroporphyrin together with traces of copro III and those materials provisionally designated hexacarboxylic and pentacarboxylic porphyrins. The slow-moving spot, which occurs only in traces is assumed to be either partially esterified porphyrin or a degradation product of the esterification. It is not completely unesterified porphyrin since that material stays firmly on the base line when run as a standard marker whereas our slow-moving material moves a short, but significant, distance from it (see Fig. 6). In practice, the spot is removed when the paper is cut after the first run. When, however, such material, collected after running several heavy applications, was bulked and run in kerosene–dioxan, it moved to the same position as uro III ester, but did not form the characteristic fan-shaped spot. Therefore, if it had been present in large amount and had not been removed completely by cutting the paper after the first run, this unknown material would have contributed to the uro III ester fraction. In practice, the risk of error from this source is negligible.

Although elution figures do not reveal 100 % recovery of the esters applied, but rather a percentage recovery which is generally of the order of 70 %, it is felt that the values obtained for standard isomer solutions by use of the correction formula are sufficiently reproducible to present a method by which the ratio of I and III isomers in a mixture may be estimated within 7 % accuracy. Agreement between the method and the results from decarboxylation and lutidine chromatography supports the efficacy of this means of estimating the ratio of uro I : uro III : pseudouroporphyrin by dioxan chromatography.

APPENDIX

Derivation of eqn. (1)

$$\text{Concentration uro III ester (g/100 ml)} = \frac{P_{405-6}}{E^{1\%}_{1cm}}$$

$$= \frac{2D_{405-6} - (D_{380} + D_{430})}{k \times E^{1\%}_{1cm}} \quad \text{(RIMINGTON AND SVEINSSON[13])}$$

$$\mu g = \frac{2D_{405-6} - (D_{380} + D_{430})}{k \times E^{1\%}_{1cm}} \times \frac{10^6 \times V}{100}$$

where:

D = extinction at wave-length indicated by subscript,
V = volume of solution (ml),
$E^{1\%}_{1cm}$ = 2300 (RIMINGTON[17]),
k = 1.6172 (derived by method of RIMINGTON AND SVEINSSON[13]).

Thus:

$$\mu g \text{ uro III ester} = \frac{[2D_{405-6} - (D_{380} + D_{430})] \times 10^6 \times V}{1.6172 \times 2300 \times 100}$$

$$= [2D_{405-6} - (D_{380} + D_{430})] \times 2.689 \times V$$

Similarly:

$$\mu g \text{ uro I ester} = \frac{[2D_{405-6} - (D_{380} + D_{430})] \times 10^6 \times V}{k \times E^{1\%}_{1cm} \times 100}$$

$$= \frac{[2D_{405-6} - (D_{380} + D_{430})] \times 10^6 \times V}{1.6051 \times 2264 \times 100}$$

$$= [2D_{405-6} - (D_{380} + D_{430})] \times 2.752 \times V$$

Therefore, in the mean:

$$\mu g \text{ uro esters} = [2D_{405-6} - (D_{380} + D_{430})] \times 2.721 \times V \qquad (1)$$

Derivation of eqn. (2)

Let x = total μg of uro ester (I + III) eluted,
y = uro ester eluted from the I position, as a percentage of the total uro ester eluted,
z = true percentage of uro I ester.

A relationship between x, y and z was required in the form, $z = f(x, y)$.

From Table IV, y was plotted against x, for z = 20 %, 25 %, 50 %, 75 %, 80 % and 90 %. 0 % and 10 % were omitted, as were 30 %, 35 % and 40 % which only had two values of x and y each. It was found that for values of x between 0.4 and 1.2, the relationship was linear. The values of a and b in the equation $y = a + bx$ were then determined for each value of z.

Using the set of equations, $y = a + bx$, values of y, for fixed values of x between 0.30 and 1.20, were found for each value of z.

y was then plotted against z for each value of x, and it was found that y and z were best related by a quadratic equation:

$$z = A + By + Cy^2 \qquad (2)$$

where A, B and C were all functions of x. z = 80 % was omitted at this stage as it did not fit in with the other figures.

Values of A, B and C were then determined for values of x between 0.30 and 1.20. Graphs of A, B and C *vs.* x were made and it was found that for values of x between 0.4 and 1.2, A, B and C were all best related to x by the following cubic equations:

$$A = 30.77 - 10.51x - 21.49x^2 + 16.04x^3$$
$$B = 0.7509 - 0.3755x + 0.5997x^2 - 0.5619x^3$$
$$C = -0.000999 + 0.005780x - 0.004834x^2 + 0.004361x^3$$

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SUMMARY

A detailed study of the chromatographic separation of uroporphyrin I and III esters by the dioxan method shows such separation to be highly sensitive to several factors. Among these, the quantity of material applied and that subsequently eluted from the paper are very important.

Technical improvements have raised the recovery by elution to about 70% (limits 50% and 100%). Application of a mathematical correction to the results leads to greater accuracy in the analysis of mixtures of isomers.

Using the method now described, it is possible to detect and estimate ratios of the isomers ranging between 17:83 and 100:0 (I:III) within 7% accuracy.

ADDENDUM

After preparing this communication, it was noted that recovery by elution was markedly improved by minimizing the time of drying the chromatograms. They are now dried at 110° for 3 min after the first run and for 7 min after the second; recoveries of between 90% and 100% are thus routinely obtained. Similar values may also be obtained by drying with a strong air blast at 50° for 10 min and 12 min respectively.

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