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GAS CHROMATOGRAPHIC ANALYSIS OF PYRROLIC ACID ESTERS FROM THE POTASSIUM PERMANGANATE OXIDATION OF BILE PIGMENTS

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SUMMARY

The NICOLAUS permanganate degradation of bile pigments and porphyrins has been adapted for use with gas chromatography in an attempt to improve the sensitivity of the method and to make it quantitative. Gas–liquid chromatographic systems for the separation of the ethyl esters of 3-methylpyrrole-2,4,5-tricarboxylic acid and 4-carboxyethyl-3-methylpyrrole-2,5-dicarboxylic acid are described. These systems have been used to estimate the content of non-IXα isomer in ox gallstone bilirubin, which is calculated to be 1–3% of the total if the non-IXα fraction is assumed to be wholly γ isomer or 2–6% if it is a β or δ isomer.

INTRODUCTION

The four isomers of biliverdin derived from the protoporphyrin ring by fission of the α, β, γ and δ bridges are shown in Fig. 1. The predominantly IXα structure assumed for natural bile pigments on the basis of early work was confirmed by GRAY *et al.*[1] using the method developed by NICOLAUS[2] for the paper chromatographic identification of pyrrole carboxylic acids formed on oxidation of naturally occurring polypyrrole pigments. Bile pigment central rings yield pyrrole 2,5-carboxylic acids on potassium permanganate oxidation. IXα isomers of biliverdin and bilirubin give 4-carboxyethyl-3-methyl-pyrrole-2,5-dicarboxylic acid (I), γ isomers 3-methylpyrrole-2,4,5-tricarboxylic acid (II). β and δ isomers and mixtures of isomers give both. This paper describes an attempt to make this technique quantitative and to improve its sensitivity by adapting it to gas–liquid chromatography (GLC). The oxidation and extraction procedure described by NICOLAUS has been slightly modified, since the visual assessment of a suitable excess of potassium permanganate gave very variable yields. Preliminary experiments were therefore directed to determining the optimum proportion of oxidant and the optimum oxidation time. This gave no markedly improved yields of pyrrolic acids, but allowed definition of a standard oxidation procedure giving reliable yields. Precipitation of oxalate in the extraction procedure has been omitted because it does not much improve the final chromatogram

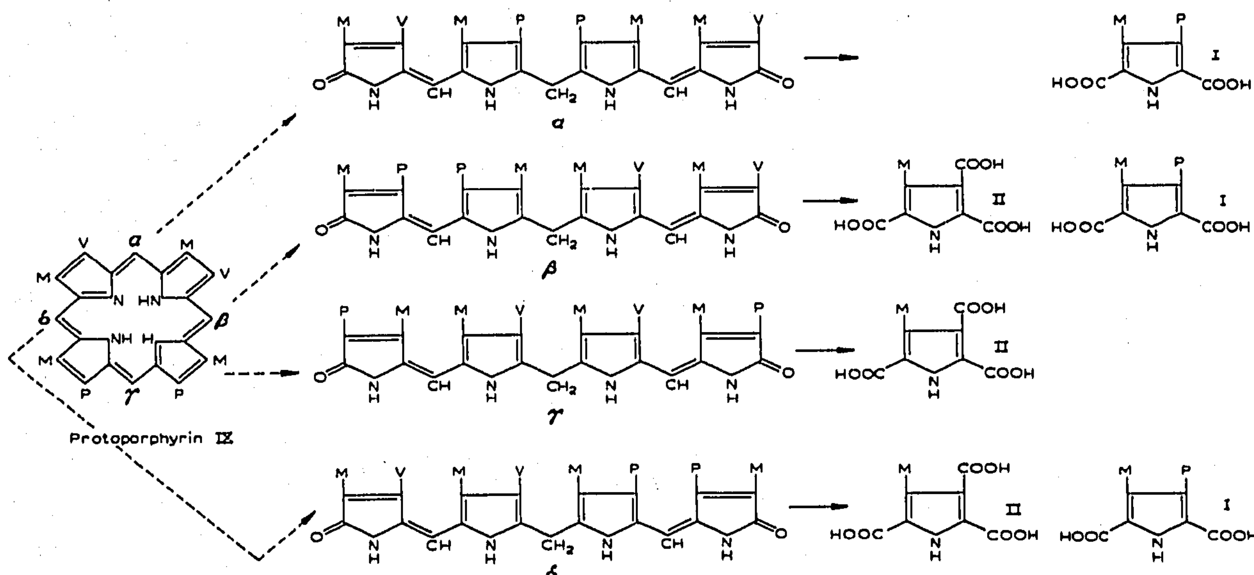

Fig. 1. The four isomeric forms of bilirubin IX and their NICOLAUS degradation products.

and causes loss of the pyrrolic acids. Pyrrolic acids decompose on melting necessitating formation of derivatives for GC. The ethyl esters are convenient because the penultimate step in pyrrolic acid synthesis is the formation of a partial ethyl ester. Final hydrolysis is wasteful causing polymerisation of the free pyrrolic acids and the partial esters are more stable for storage. The method of final esterification must be applicable to imprecisely known quantities of free pyrrolic acids in bile pigment oxidation mixtures. Of diazoethane, 5% ethanolic sulphuric acid and ethanolic boron trifluoride, the last two esterification reagents were unsuitable, causing polymerisation of free pyrrolic acids. Pyrrolic acid (II) in a moderate excess of diazoethane formed the triester triethyl-3-methylpyrrole-2,4,5-tricarboxylate (III); a larger excess esterified the imino hydrogen as well forming triethyl-1-ethyl-3-methylpyrrole-2,4,5-tricarboxylate (IV). The imino hydrogen of (I) was unaffected by large excesses of diazoethane, only diethyl-4(2)-carboxyethyl-ethyl-3-methylpyrrole-2,5-dicarboxylate (V) being formed. The method developed for the separation and identification of (III), (IV) and (V) has been used to estimate the proportion of non-IXα isomer in natural (ox gallstone) bilirubin.

MATERIALS AND METHODS

Chemicals

Ox gallstone bilirubin and protoporphyrin were obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks. Haemin was prepared from whole human blood by the method of NASHIDA AND LABBE[3].

The pyrrolic acid esters (III), (IV) and (V) for use as GLC standard were prepared from the diethyl ester of (II) 3,5-diethoxycarbonyl-4-methylpyrrole-2-carboxylic acid and the monoethyl ester of (I) 3(2-carboxyethyl)-5-ethoxycarbonyl-3-methylpyrrole-2-carboxylic acid. The diester of (II) was prepared by reacting ethyl aceto-acetate with glacial acetic acid and sodium nitrite and condensing the resulting

oxime with ethyl oxaloacetate[4]. The monoester of (I) is available by the route described by FISCHER AND ORTH[5]. We are grateful to Dr. R. A. NICOLAUS and to Dr. A. H. JACKSON for samples of this compound. (IV) and (V) were prepared by treating the diester of (II) and the monoester of (I) with a large excess of diazoethane. (III) was prepared by treatment of the diester of (II) with 5% ethanolic sulphuric acid for 24 h. The crude preparations of (III), (IV) and (V) were purified on 25 ml columns of silicic acid, conditioned at 110° for 24 h before use. The columns were made up in light petroleum (b.p. 40–60°) and washed with 50 ml of light petroleum before application of the samples in the minimum quantity of methanol. The pyrrolic acid esters were eluted from the columns as the proportion of ether in the column washings was increased. The emergent fractions were scanned for pyrrolic acid ester content by measuring the absorption spectra from 200–400 mμ in a Unicam SP. 800 UV spectrophotometer. Fractions so located were then further scanned by GLC and chromatographically pure fractions were retained for use as standards. The melting points and C, H and N analysis values of the standard preparations of pyrrolic acid esters are given in Table I.

TABLE I

THE MELTING POINTS, AND C, H AND N ANALYSIS VALUES OF THE STANDARD PYRROLIC ACID ESTER PREPARATIONS

Pyrrolic acid ester	*Description*	*Melting point (°C)*	*UV maxima (mμ)*	*C%*		*H%*		*N%*	
				Observed	*Theoretical*	*Observed*	*Theoretical*	*Observed*	*Theoretical*
III	white solid	140	222 275	55.3	56.6	6.4	6.3	—	4.7
IV	white oil	—	225 260	59.2	59.0	7.1	7.0	4.4	4.3
V	white solid	185	222 277	58.8	59.0	7.2	7.0	4.2	4.3

Storage of standard solutions of pyrrolic acid esters

To avoid the error and wastage inherent in repeatedly weighing small quantities of standard compounds, a solution was prepared and divided into small aliquots which were dried under a current of nitrogen and stored at 0° for use as required. 2.8 mg, 2.6 mg and 3.3 mg of (III), (IV) and (V), respectively were transferred to a 5 ml volumetric flask and dissolved in ethanol (spectroscopic grade: Hopkin and Williams Ltd.). 100 μl portions of this solution were distributed into 48 acid washed vials, dried and stored at 0°.

Preparation of a calibration curve

100 μl of spectroscopic ethanol or chloroform was added to one of the vials containing known quantities of the three standard solutions giving a solution containing 0.56, 0.52 and 0.66 μg/μl of (III), (IV) and (V), respectively. The vial was closed immediately with a rubber injection septum to minimise evaporation. The detector responses in mm^2 at noted attentuation and chart speed were measured for

1, 2, 5 and 10 μl quantities of this solution; all measurements were made with 1 and 10 μl Hamilton microsyringes.

Potassium permanganate oxidation of bile pigments and porphyrins

The oxidation and extraction procedure described by NICOLAUS was modified slightly since errors in determining a suitable excess of potassium permanganate visually allowed considerable variation in the quantity of oxidant added. Theoretically, 22 and 19 oxygen atoms, respectively are required to degrade one molecule of protoporphyrin or bilirubin to four pyrrole rings with the vinyl side chains oxidised to carboxyl groups, so that one mole of protoporphyrin would require 22 $\times \frac{2}{3}$ moles of alkaline potassium permanganate, and one mole of bilirubin 19 $\times \frac{2}{3}$ moles of alkaline potassium permanganate. The optimum proportion of oxidant was found to be 2 molar equivalents of alkaline potassium permanganate. For 1 mg of protoporphyrin or 1 mg of bilirubin 0.113 ml saturated potassium permanganate and 0.107 ml saturated potassium permanganate, respectively are required since saturated potassium permanganate contains 6.3 g/100 ml at 20°. The optimum oxidation time was between 2 and 5 min and the yield was improved if ten times the volume of 1 *N* potassium carbonate recommended by NICOLAUS is used.

Samples of pigment from 10–100 mg were dissolved in 2–20 ml of 1 *N* potassium carbonate and two molar equivalents of saturated potassium permanganate (as previously defined) added fairly rapidly and dropwise to the well-shaken solution. After 5 min excess oxidant was destroyed with a minimum quantity of sodium *meta*-bisulphite and manganese dioxide removed by filtration. The precipitate and the reaction vessel were rinsed with 2–20 ml of hot distilled water and the washings added to the filtrate. The cooled filtrate was washed with 2 $\times$ (5–20) ml of peroxide-free ether before acidification and extraction into 5 $\times$ (5–20) ml of ether. The extract was reduced to 10 ml before esterification.

The esterification of pyrrolic acid extracts with diazoethane

Freshly made diazoethane was added to the ether extracts of the pyrrolic acids until there was a visible excess. The extracts were left in the dark at 4° for 16 h. Excess diazoethane was removed by the addition of a few drops of glacial acetic acid. The extracts were washed with sodium hydrogen carbonate and then with distilled water, evaporated in a current of air and dried in a vacuum desiccator.

Preparation of columns and column packings

Diatoport S 60–80 mesh as supplied by Hewlett Packard was impregnated with stationary liquid by the method of PARCHER AND URONE[6]. Newly packed columns were conditioned at 250° for about 64 h before use.

RESULTS AND DISCUSSION

The choice of GLC conditions for the analysis of pyrrolic acid esters

The Pye Panchromatograph with argon ionization detector was used in preliminary work. Samples were evaporated onto stainless steel gauzes for application to the columns. In later work using a Pye 104 gas chromatograph with flame ionization detector samples were applied by liquid injection. Acid washed Chromosorb P was

used for column packings with a high percentage of stationary phase, and silanised Diatoport S for columns with a low loading of stationary phase. A moderate mesh size (60–80) has been used for all columns.

The use of four stationary liquids has been investigated. These were Apiezon L (APL); the methyl silicone gum SE-30; poly(diethylene glycol succinate) (DEGS); and poly(neopentyl glycol adipate) (NGA). Samples of (III), (IV) and (V) were run on 5 ft. × ¼ in. 10% columns at temperatures from 150° to 225° at argon flow rate 100 ml/min. The two non-polar columns were unsatisfactory, giving poor resolution and badly tailing peaks, but on the polar columns the compounds were well separated with symmetrical peaks. Initially 5 ft. × ¼ in. columns of 1% NGA and DEGS on Diatoport S, 60–80 mesh were used at 200° and flow rates of 100 ml/min, for argon on the Panchromatograph, and 50/50/500, for argon, hydrogen and air, respectively on the Pye 104. These were adequate for the identification of (V) in the oxidation product of 1–2 mg of bilirubin and of (III), (IV) and (V) from 3–4 mg of protoporphyrin. The oxidation of 50 mg of pigment provides sufficient material for 10 GLC samples. Modifications of this system were necessary to investigate the non-IXα content of natural bilirubin since the pyrrolic acids are only minor products of the permanganate oxidation, and greater resolution is required if the area of the chromatogram where ester IV emerges is not to be obscured at high sensitivities by tailing early-emerging components. The most effective isothermal system was a 15 ft. × ¼ in. column at 200° with flow rates of 15/15/500 for argon, hydrogen and air, respectively. Resolution was further increased by temperature programming. The best resolution of (IV) from esterified oxidation extracts was obtained on 15-ft. columns of 1% NGA and DEGS programmed from 150 to 200° at 1°/min with flow rates

TABLE II

THE RETENTION TIMES OF (III), (IV) AND (V) UNDER VARIOUS CONDITIONS OF GLC

Retention time (min)			*Temperature (°C)*	*Column*	*Argon flow rate (ml/min)*
III	*IV*	*V*			
9.5	6.8	14	195	1% NGA	50
3.1	2.3	4.4	215	5 ft.	
44	27	60	195	10% NGA	100
22	15	32	210	5 ft.	
14	11	21	220		
40	29	60	194	1% NGA	15
13	9.9	19	220	15 ft.	
18	13	30	197	1% NGA	50
7.2	5.3	10	220	15 ft.	
7.9	4.4	10	195	1% DEGS	50
2.9	1.7	3.6	215	5 ft.	
21	12	27	190	10% DEGS	100
13	7.7	16	204	5 ft.	
6.7	4.1	8.3	222		
35	19	45	193	1% DEGS	15
11	6.4	13	220	15 ft.	
14	7.6	18	195	1% DEGS	50
4.8	2.9	5.9	220	15 ft.	

15/15/500 for argon, hydrogen and air, respectively. The retention times of (III), (IV) and (V) under various conditions of GLC are summarised in Table II.

The qualitative identification of the pyrrolic acid esters

Identification of the pyrrolic acid esters were based wherever sample size would permit, on comparison with the retention times of standard pyrrolic acid esters run under the same conditions on both NGA and DEGS. It was usually simple to identify (V), the product of the α isomers of bile pigments. To identify very small peaks of (IV) and (III) in esterified oxidation extracts from natural bilirubin, samples of mixed standards and unknowns were run to see if the tentatively identified peaks emerged with the standard compounds. To minimise the risk of misidentification (III) and (IV) were identified by their retentions relative to (V) on DEGS and NGA. The ratio of the adjusted retention times of any two solutes, *i.e.* the intervals between the emergence of an unabsorbed gas and the solutes, depends only on the operating temperature and the stationary phase. Table III records the retentions of (III) and (IV) relative to (V) for the conditions used.

TABLE III

THE GLC RETENTIONS OF (III) AND (IV) RELATIVE TO (V)

Stationary liquid	*Temperature (°C)*	*Retention time of (IV)/retention time of (V)*			*Retention time of (III)/retention time of (V)*		
		No. of observations	*Mean value*	*Standard deviation*	*No. of observations*	*Mean value*	*Standard deviation*
NGA	195	9	0.67	0.93×10^{-2}	9	0.47	1.0×10^{-2}
	198	19	0.67	0.4×10^{-2}	19	0.48	1.0×10^{-2}
	200	22	0.67	0.67×10^{-2}	22	0.48	0.58×10^{-2}
	150–200 at 1°/min	7	0.78	0.85×10^{-2}	7	0.64	0.9×10^{-2}
DEGS	195	15	0.77	0.71×10^{-2}	15	0.42	0.71×10^{-2}
	198	4	0.78	0.5×10^{-2}	4	0.43	0.5×10^{-2}
	200	26	0.78	0.8×10^{-2}	26	0.44	1.3×10^{-2}
	150–200 at 1°/min	5	0.85	0.9×10^{-2}	5	0.63	1.3×10^{-2}

Quantitative use of the GLC method

Calibration curves were prepared before each series of oxidations. From this data the micromolar response factors for (III), (IV) and (V) at a standard attenuation (y) and chart speed (t) may be determined as follows:

$$\mu F_r = \frac{\text{sample size } (\mu\text{moles})}{\text{observed peak area}} \times \frac{y't}{yt'}$$

when y' and t' are the attentuation and chart speed at which the peak area was measured. The quantity of material in an unknown sample is determined by multiplying the observed peak area, corrected to attentuation y and chart speed t, by the response factor. To determine the proportion of isomers in a sample of bile pigment the response factors relative to the response factor for (V) were calculated. The relative molar response factors (f_{III}, f_{IV} and f_{V}) are therefore:

$f_{III} = \mu F_{rIII}/\mu F_{rV}$
$f_{IV} = \mu F_{rIV}/\mu F_{rV}$
$f_V = 1$

The response of the flame ionization detector to four different quantities of a standard solution of the pyrrolic acid esters (III), (IV) and (V) was measured on thirteen different occasions under different GLC conditions. Visual inspection of the values obtained from these data suggested that, despite the use of different sample quantities, changes in the chromatographic conditions and daily variations of the equipment, f_{III}, f_{IV} and f_V were sufficiently close for the mean values to be used in calculating analytical results without continuous calibration of the detector response. This was formally tested using the statistical analysis of variance model. The method adopted was to determine whether a statistically significant proportion of the total variation in the values for the relative molar response factors originated from daily variation or from variation in sample size. If the hypothesis that (a) sample size and (b) day and conditions of the experiment have no effect on results is true, then the calculated statistic has an "F" distribution so that, with the choice of an appropriate level of significance (5% and 2.5%), the hypothesis is rejected if the calculated statistic is greater than the tabulated value for the cumulative "F" distribution with the relevant degree of freedom. However, if these tests show that the hypotheses cannot be rejected then it can be concluded that the mean values of the response factors can be used in calculations. Alternatively, if the hypotheses are rejected then it must be concluded that owing to variation in sample size and/or conditions of use the chromatograph will have to be calibrated before each series of analyses.

The hypotheses under test may be formally expressed as:

1st. null hypothesis (H_0^1). The effect of the different sample sizes is not significant. Hypothesis to be accepted if the estimated variance ratio (F^1) with 3 and 36 degrees of freedom $\leqslant F_{5\%}$ and $F_{2.5\%}$ (3,36).

2nd. null hypothesis (H_0^2). The effect of the day and/or condition of the experiment is not significant. Hypothesis to be accepted if the estimated variance ratio (F^2) with 12 and 36 degrees of freedom $\leqslant F_{5\%}$ and $F_{2.5\%}$ (12,36)

	5%	2.5%
F (3,36)	2.87	3.51
F (12,36)	2.03	2.33

Results. (1) For $f_{III} - F^1 = 0.529 < F_{5\%}$ (3,36) therefore accept H_0^1; for $f_{IV} - F^1 = 1.18 < F_{5\%}$ (3,36) therefore accept H_0^1. (2) For $f_{III} - F^2 = 14.5 < F_{2.5\%}$ (12,36) therefore reject H_0^2; for $f_{IV} - F^2 = 12.55 < F_{2.5\%}$ (12,36) therefore reject H_0^2. For both F_{III} and f_{IV} the variation in quantities did not significantly affect the results at the 5% and 2.5% significance levels, whereas the occasion and/or GLC conditions when the readings were taken did significantly affect the results. This means that although the detector response to varying quantities of each of the pyrrolic acid esters is linear (on thirteen occasions for the range observed), the ratios between the gradients of the calibration curves of (III), (IV) and (V) vary with day and/or GLC conditions so that to estimate the proportions of pyrrolic acid esters in a sample the detector must be recalibrated before each series of analyses. The relative molar response factors are also available from the gradients m_{III}, m_{IV} and m_V of the

TABLE IV

THE MEAN VALUES OBTAINED FOR THE RELATIVE MOLAR RESPONSE FACTORS OF (III) AND (IV) ON THIRTEEN SEPARATE OCCASIONS UNDER VARIOUS GLC CONDITIONS

Column	*Argon flow rate*	*Temperature (°C)*	f_{III}	f_{IV}
1% NGA 5 ft.	50 ml/min	200	1.0725	1.0025
1% NGA 5 ft.	50 ml/min	200	1.0825	1.0125
1% NGA 5 ft.	15 ml/min	200	1.0275	0.88
1% NGA 15 ft.	15 ml/min	200	1.0575	1.005
1% NGA 15 ft.	15 ml/min	200	1.0325	1.9925
1% DEGS 5 ft.	50 ml/min	200	1.0675	1.9825
1% DEGS 5 ft.	50 ml/min	200	1.055	0.9975
1% DEGS 5 ft.	50 ml/min	200	1.0675	0.9875
1% DEGS 5 ft.	15 ml/min	200	1.065	0.9025
1% DEGS 15 ft.	15 ml/min	200	1.0625	0.9025
1% DEGS 15 ft.	15 ml/min	200	1.0725	1.0
1% NGA 15 ft.	15 ml/min	150–200 at 1°/min	1.155	0.98
1% DEGS 15 ft.	15 ml/min	150–200 at 1°/min	1.0625	0.9875

calibration curves ($y' = m'x'$ when y' is the observed peak area and x' is the sample concentration in moles) of (III), (IV) and (V), respectively.

$$f_{III} = m_V/m_{III} \qquad f_{IV} = m_V/m_{IV}$$

However for calculations in the present work the appropriate values for f_{III} and f_{IV} are estimated as the arithmetic means of the values obtained from four different quantities of the standard solution of (III), (IV) and (V), on the relevant day. These values are collected in Table IV.

The percentage molar composition of a sample is calculated from the relevant relative response factors and observed peak areas in the following way. If a portion of sample containing a moles of (III), b moles of (IV) and c moles of (V) gives peak areas A, B and C mm² at the same attenuation and chart speed for (III), (IV) and (V), respectively, then:

$$a:b:c = Af_{III}:Bf_{IV}:C$$

and the sample therefore contains:

$100Af_{III}/(Af_{III} + Bf_{IV} + C)\%$ of (III)
$100Bf_{IV}/(Af_{III} + Bf_{IV} + C)\%$ of (IV)
$100C/(Af_{III} + Bf_{IV} + C)\%$ of (V)

The relative stabilities of the pyrrolic acids to the oxidation, esterification and extraction procedure

In order to calculate the proportion of isomers present in a sample of bile pigment from a chromatogram of the esterified oxidation products, the relative stabilities of the pyrrolic acids and esters to the oxidation, esterification and extraction procedure must be known. Since it has not been possible to synthesise 3-methyl-4-vinylpyrrole-2,5-dicarboxylic acid, the precursor of (II), direct recovery measurements have not been possible. The relative stabilities of the pyrrolic acids have therefore been deduced from an examination of the oxidation products of a ring compound such as protoporphyrin, haemin or haematin, assuming that permanganate oxidation of these compounds result in random ring fission yielding equal quantities of 3-methyl-4-vinylpyrrole-2,5-dicarboxylic acid and (I). On oxidation and esterification with excess diazoethane, therefore, $2k$ moles of haemin will yield, $r_{IV}k/100$ moles of (IV) and $r_{V}k/100$ moles of (V), if r_{IV} and r_{V} are the overall percentage molar recoveries of (IV) and (V). If a portion of an esterified oxidation extract gives peak areas B_1 and C_1 mm² at constant attentuation and chart speed for (IV) and (V), then

$$r_{IV}k/100 : r_{V}k/100 = B_1 f_{IV} : C_1$$

if f_{IV} is the relevant response factor.

TABLE V

THE VALUES OBTAINED FOR K, THE FACTOR FOR THE RECOVERY OF PYRROLIC ACID ESTER (IV) RELATIVE TO (V)

No.	*Starting material*	*GLC conditions*	B_1	C_1	f_{IV}	K
1	haematin	15 ft. 1% NGA 150–200 at 1°/min.	360	840	0.98	0.42
2	haematin	see (1)	125	514	0.98	0.43
3	haematin	see (1)	55	121	0.98	0.45
4	haematin	15 ft. 1% DEGS 150–200 at 1°/min	156	352	0.9875	0.44
5	haematin	see (4)	108	237	0.9875	0.45
6	protoporphyrin	15 ft. 1% DEGS 200°	108	2000	0.9975	0.205
7	protoporphyrin	see (6)	725	1680	0.98	0.425
8	protoporphyrin	5 ft. 1% NGA 200°	540	1260	0.99	0.43
9	protoporphyrin	5 ft. 1% DEGS 200°	505	1060	0.9875	0.47
10	protoporphyrin	15 ft. 1% DEGS 200°	116	267	0.99	0.43
11	haemin	15 ft. 1% NGA 200°	232	520	1.005	0.45
12	haemin	15 ft. 1% DEGS 200°	217	450	0.9025	0.44
13	protoporphyrin	5 ft. 1% DEGS 200°	135	305	0.9975	0.44
					Mean = 0.44 ± 0.013	

$$
\begin{aligned}
r_{IV}:r_V &= B_1 f_{IV}:C_1 \\
&= B_1 f_{IV}/C_1:1 \\
&= K:1 \\
K &= r_{IV}/r_V
\end{aligned}
$$

Observations of the relative yields of (IV) and (V) from protoporphyrin, haemin and haematin under various conditions of chromatography, are collected in Table V. Experiments in which (III) was formed have been excluded from consideration because of the difficulty of estimating the total recovery of 3-methyl-4-vinylpyrrole-2,5-dicarboxylic acid from varying mixtures of two products. The calculations assume an excess of diazoethane during esterification.

Calculation of the percentage of isomers in a sample of bile pigment

If a sample of bile pigment contains $a\%$ γ isomer and $(100-a)\%$ α isomer and a portion of the esterified oxidation extract gives peak areas B_2 and C_2 mm² at common attenuation and chart speed for (IV) and (V), respectively, then

$$\frac{r_{IV}}{100}\cdot(100-a) : \frac{r_V}{100}\cdot a = B_2 f_{IV} : C_2$$

and

$$(100-a):Ka = C_2:B_2 f_{IV}$$

if f_{IV} is the appropriate response factor.

TABLE VI

THE PERCENTAGE CONTENT OF NON-IXα ISOMERS IN OX GALLSTONE BILIRUBIN ESTIMATED BY GLC ANALYSIS OF ESTERIFIED EXTRACTS OF THE PRODUCTS OF POTASSIUM PERMANGANATE OXIDATION

Sample No.	*GLC conditions*	C_2	B_2	f_{IV}	*percentage content of non-IXα isomer if it is assumed γ*	*percentage content of non-IXα isomer if it is assumed β or γ*
1	15 ft. 1% NGA 200°	10900	96	1.005	1.97	3.94
		9450	80	1.005	2.1	4.2
		6650	56	1.005	1.9	3.8
		17200	127	1.005	1.63	3.26
		13500	80	1.005	1.38	2.76
1	15 ft. 1% DEGS 200°	14700	85	0.9025	1.18	2.36
		27200	169	0.9025	1.25	2.5
		35000	255	0.9025	1.54	3.08
		1900	130	0.9025	1.39	2.78
2	15 ft. 1% DEGS 200°	15900	144	0.9025	1.33	2.66
3	15 ft. 1% DEGS 200°	17000	80	0.98	1.02	2.04
		13900	240	0.98	3.7	5.5
4	15 ft. 1% DEGS 200°	12300	54	0.98	0.97	1.84
5	15 ft. 1% NGA 200°	28000	144	0.9925	1.14	2.28
6	15 ft. 1% NGA 150–200° at 1°/min	3600	20	0.9875	1.18	2.36
		9300	48	0.9875	1.14	2.28
		680	10	0.9875	3.2	6.4
6	15 ft. 1% DEGS 150–200° at 1°/min	3500	43	0.986	2.7	5.4

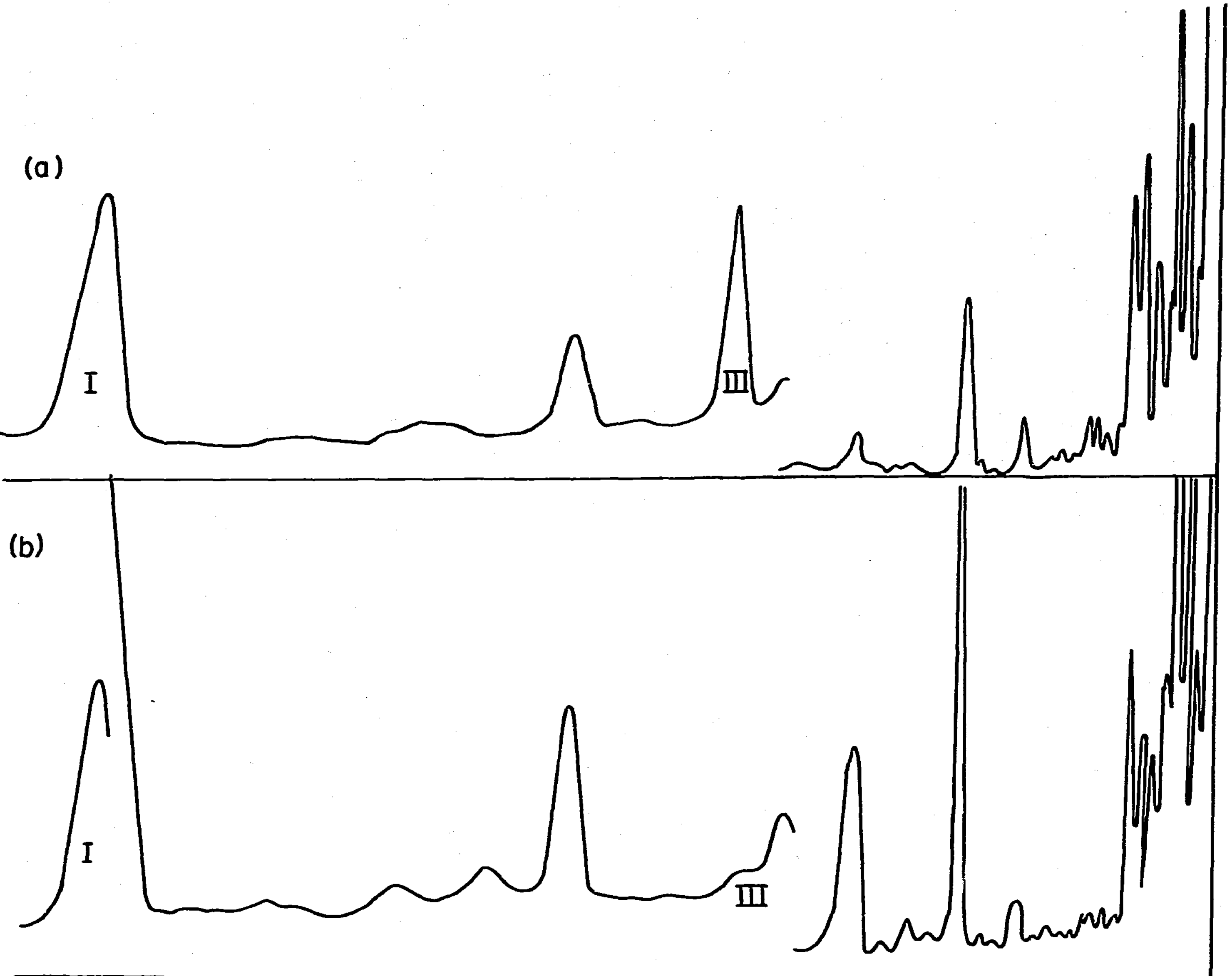

Fig. 2. Chromatograms on 15 ft. 1% DEGS at 200°C, flow rates 15/15/500, chart speed ½ in./min (scale 6 mm = 1 in.). (a) esterified extract of protoporphyrin oxidation product; (b) esterified extract of bilirubin oxidation[4].

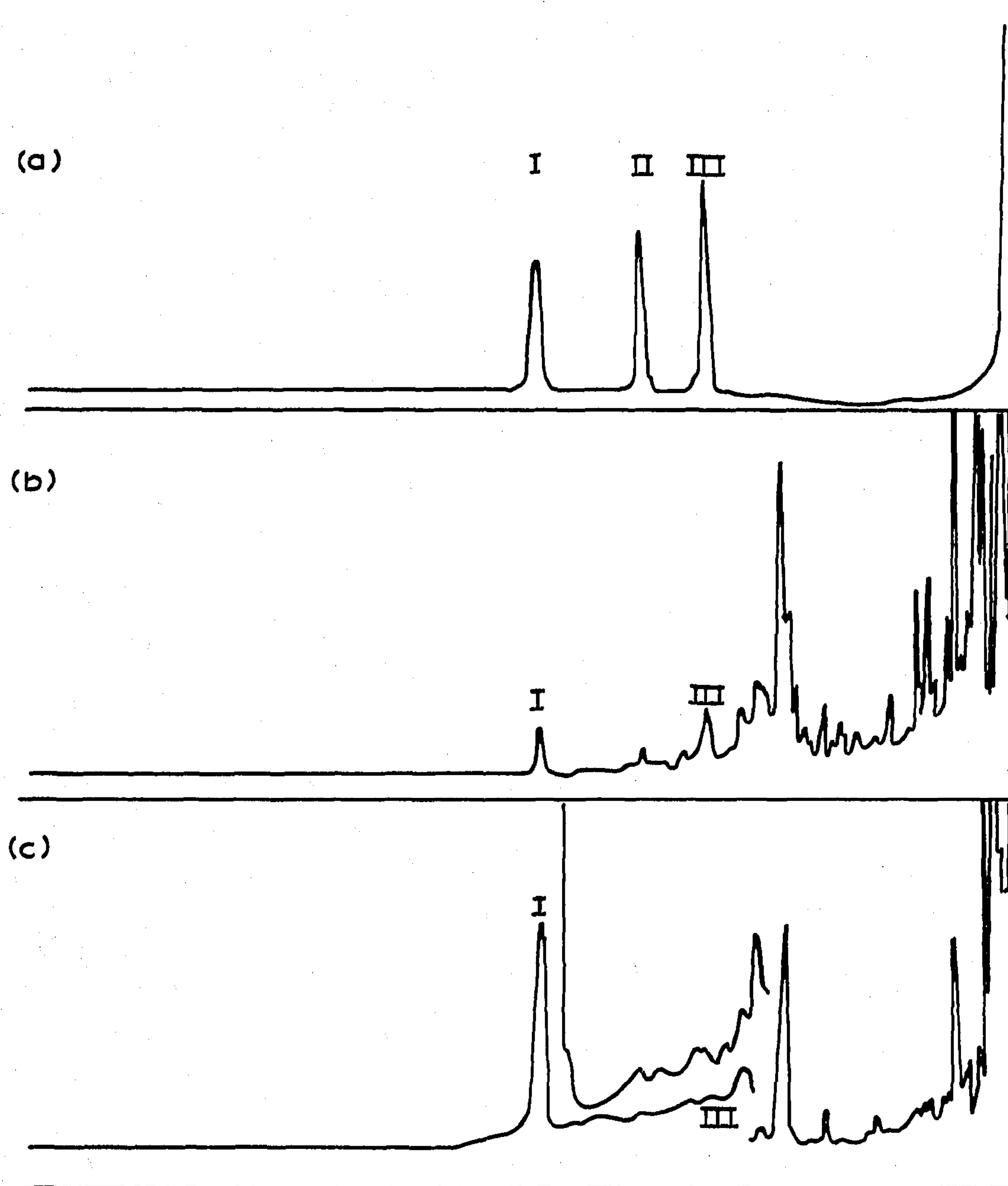

Fig. 3. Chromatograms on 15 ft. 1% NGA, programmed from 150 to 200°C at 1°/min, flow rates 15/15/500, chart speed 12 min/in. (scale 6 mm = 1 in.). (a) esterified pyrrolic acid esters; (b) esterified extract of haematin oxidation[1]; (c) esterified extract of bilirubin oxidation[6].

$$KC_2a = B_2f_{\text{IV}}\,(100 - a)$$
$$a(KC_2 + B_2f_{\text{IV}}) = 100B_2f_{\text{IV}}$$

The percentage of γ pigment is therefore given by

$$a = 100B_2f_{\text{IV}}/(KC_2 + B_2f_{\text{IV}})\%$$

If the sample of bile pigment contains a% β or δ pigment, or if a% of the sample

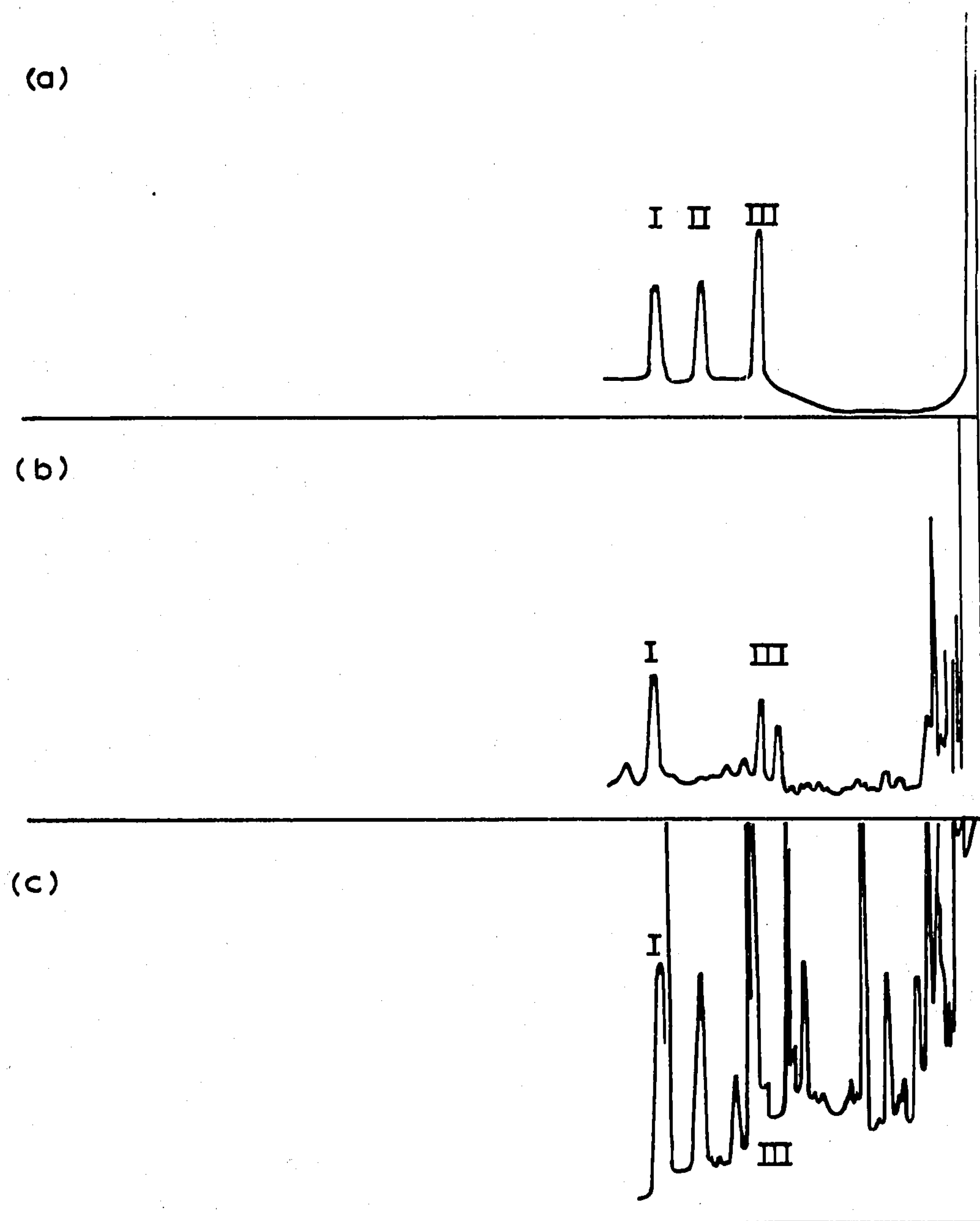

Fig. 4. Chromatograms on 15 ft. 1% DEGS, programmed from 150 to 200°C at 1°/min, flow rates 15/15/500, chart speed 12 min/in. (scale 6 mm = 1 in.). (a) Standard pyrrolic acid esters; (b) esterified extract of haematin oxidation[1]; (c) esterified extract of bilirubin oxidation[6].

consists of a fraction derived from random fission of a porphyrin ring, and $(100-a)\%$ is derived from pure α fission then on oxidation the non-IXα fraction will form $a/2\%$ 3-methyl-4-vinylpyrrole-2,5-dicarboxylic acid and $a/2\%$ (V) so that:

$$(100-a+a/2):Ka/2 = C_2:B_2f_{IV}$$

or

$$(100-a/2):Ka/2 = C_2:B_2f_{IV}$$

The percentage of non-IXα pigment will be given by

$$a = 200 B_2 f_{IV} / (KC_2 + B_2 f_{IV}) \%$$

The estimation of the percentage content of non-IXα isomers in ox gallstone bilirubin

The results obtained from the attempted measurement of the isomeric content of natural bilirubin are collected in Table VI. 10–100 mg quantities of bilirubin were oxidised, extracted and esterified as described. The residues were dissolved in chloroform 10–100 μl for injection in 1-μl quantities. The peak for (IV) is small in relation to (V) and is poorly resolved from the preceding material as can be seen in Figs. 2, 3 and 4. The peak areas recorded are therefore at best estimated judging the peak base by eye.

Discussion

Small quantities of non-IXα isomer have been detected in natural bilirubin from ox gallstones by GLC analysis of the products of potassium permanganate oxidation. This confirms the original observations of PETRYKA[7]. If the non-IXα fraction is wholly γ isomer it is estimated that it would account for between 1 and 3% of the total bile pigment; if it is a β or δ isomer it would account for between 2 and 6% of the total pigment. Difficulties in measurement of trace peaks and incomplete resolutions render such estimates inaccurate. Moreover, their calculation involves an assumption that cyclic tetrapyrroles afford equal quantities of the initially formed pyrrolic acids in oxidation. The resulting values are the same as those noted by NICHOL AND MORELL[8] in mass spectrometric investigations, but are much higher than the values of 0.1% β isomer and 0.3% δ isomer detected in pig bile bilirubin by O'CARRA AND COLLERAN[9]. These differences may be attributable to species differences but the interpretation of the mass spectrometric analysis of bile pigment isomers has been disputed[9]. NICHOL AND MORELL claim that the product of ascorbate/O_2 oxidation of pyridine haemochrome is a pure β and δ biliverdin whereas RÜDIGER[10] and O'CARRA AND COLLERAN[9] have shown it to be a mixture of all four isomers. These workers have developed techniques for the thin-layer chromatographic separation of all four verdin isomers. The separated isomers can then be further identified by their pyrrole dialdehyde products on dilute sodium dichromate oxidation[10]. In addition O'CARRA AND COLLERAN have been able to distinguish β and δ isomers by comparison with a synthetic β bile pigment.

This adaptation of the NICOLAUS method to GLC, although not achieving greater sensitivity, has made quantitation possible allowing estimation of the relative recovery of the pyrrolic acids. The results indicate that (IV) is about half as stable as (V) to the conditions of oxidation and extraction. Earlier work based on qualitative interpretation of paper chromatograms may, therefore, have led to misleading results.

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