composition of each of the three fractions thus obtained has been studied separately and the final possible glyceride composition computed from the resulting data.

4. The glyceride composition of panther fat, unlike that of the fat of certain other land animals, closely follows Hilditch's (1956) rule of 'even' distribution.

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5-Formylfuroic Acid and the Carbazole Reaction for Uronic Acids and Acidic Polysaccharides

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It has been shown (Bowness, 1957) that furfuraldehyde and 5-hydroxymethylfurfuraldehyde in sulphuric acid do not give any substantial coloration with carbazole under the conditions of the procedure (Dische, 1947) for estimating uronic acids.

In the present work it was found that two other derivatives of furan, 5-formylfuroic acid (I) and 2:5-diformylfuran (II), both give intense colorations under these conditions, and also under the conditions of the modified carbazole reaction which Dische (1950) showed would distinguish between various polyuronides. It appears probable that formylfuroic acid is the principal agent responsible for the formation of colour from uronic acids in these two procedures.

MATERIALS AND METHODS

Materials

5-Formylfuroic acid (m.p. 195°) and 2:5-diformylfuran (m.p. 107°) were prepared and purified by the methods of Cooper & Nuttall (1912). Methyl β -D-glucofuranuron-

lactone (m.p. 135°) and 1:2-O-isopropylidene- α -D-glucofuranuronolactone (m.p. 116°) were prepared by the methods of Owen, Peat & Jones (1941). The sulphuric acid for the reaction at 60° (Dische, 1950) was obtained by adding 6 vol. of H_2SO_4 (A.R., Hopkin and Williams Ltd.) cooled to 0°, to 1 vol. of water in an ice bath. Heparin (Pularin) was obtained from Evans Medical Supplies Ltd. The sources or methods of preparation of all other reagents have been described previously (Bowness, 1957).

Methods

Reaction at 100°. Details of this procedure have been described in a previous paper (Bowness, 1957).

The absorption spectra recorded in Fig. 1 and the absorption values in Table 1 were obtained with a Hilger Uvispek (H. 700) spectrophotometer. Measurements were made 2.0–2.75 hr. after removing the sulphuric acid solutions from the boiling water. The solutions without carbazole were read against a sulphuric acid blank; those with carbazole were read against the blank containing carbazole.

Reaction at 60°. The procedure of Dische (1950) was employed, except that 0.2 ml. of a 0.3% (instead of a 0.1%) (w/v) ethanolic solution of carbazole was used. Absorption spectra (Fig. 2 and Table 2) were obtained as described above for the reaction at 100°. Measurements were made 0.5–1.25 hr. after removal of the solutions from the water bath.

RESULTS

Reaction at 100°

In the presence of carbazole. Table 1 shows that furfuraldehyde and 5-hydroxymethylfurfuraldehyde do not give appreciable colours in the reaction

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with carbazole at 100° in sulphuric acid (cf. Bowness, 1957). Galacturonic acid, glucuronolactone, all derivatives and compounds of glucuronic acid tested in this work (not shown in Table 1, but listed in Table 2) and 5-formylfuroic acid gave a substantial coloration with a principal absorption maximum at, or near, 525 m μ and a subsidiary maximum at 395 m μ . 2:5-Diformylfuran also gave a strong colour with carbazole, but the absorption maxima, at 550 and 430 m μ , were quite distinct from those given by formylfuroic acid and the uronic acids. The absorption spectra in the visual region given in the test by glucuronolactone (Fig. 1), galacturonic acid and formylfuroic acid were all of the same shape, though the molar intensities of absorption for the complex with formylfuroic acid were considerably higher than for the other two.

Reaction in the absence of carbazole. The absorption curve of formylfuroic acid in sulphuric acid without carbazole differed slightly from that for glucuronolactone (Fig. 1) and from those for other uronic acid derivatives, but the principal absorption band shown by all these compounds was in the region 298–308 m μ and there was no subsidiary band near 224 m μ as there was for 2:5-diformylfuran.

Glucosaccharic acid heated with concentrated sulphuric acid slowly forms furan-2:5-dicarboxylic acid (Tollens & Yoder, 1901). This last compound was almost certainly responsible for the bottom curve in Fig. 1 ($\lambda_{\max}$, 272 m μ) obtained with calcium saccharate. The absorption intensity increased considerably when the time of heating was prolonged. On mixing with 3 vol. of water the absorption maximum of this solution approached that observed by Hausser, Kuhn, Smakula & Deutsch (1935). No coloration was obtained when carbazole was added to this solution, even after standing for 24 hr.

A solution of 5 mg. of glucuronolactone in sulphuric acid, after treatment under the standard conditions of the reaction at 100°, was mixed with 8 vol. of water. Three-quarters of the acid was

neutralized with sodium carbonate and the mixture was extracted twice with 50 ml. of diethyl ether. The ether extract was dried over anhydrous sodium sulphate. The absorption spectrum of the ether extract was very similar to that obtained with a solution of 5-formylfuroic acid in diethyl ether. The positions of the absorption maxima were identical (275 m μ), but the positions of the absorption minima, below 300 m μ , and the ratios of maximum to minimum were somewhat different. The recovery of 5-formylfuroic acid in the ether extract (as estimated by the absorption intensities at $\lambda_{\max}$) was only about 10 % of that in the usual procedure for the reaction at 100°.

Fig. 1. Absorption spectra of solutions in sulphuric acid, under the conditions of the carbazole reaction at 100°, with (350–650 m μ) and without (220–350 m μ) added ethanolic carbazole. Each solution contained 6 ml. of sulphuric acid and 1 ml. of an aqueous solution of one of the substances listed below; the added carbazole was 0.2 ml. of a 0.2% ethanolic solution. A, Glucuronolactone (100 μ g.); B, furfuraldehyde (100 μ g.); C, 5-formylfuroic acid (100 μ g.); D, calcium D-saccharate (120 μ g.).

Table 1. *Intensities of absorption of light, and wavelength maxima, of substances in sulphuric acid in the reaction at 100° with and without carbazole*

Procedural details are described under Methods; 0.715 μ mole of each substance was tested. Figures in parentheses refer to secondary absorption maxima.

Substance	Without carbazole, in the range 220–350 m μ		With carbazole, in the range 360–650 m μ	
	$\lambda_{\max}$	$E_{\max}$	$\lambda_{\max}$	$E_{\max}$
5-Formylfuroic acid	298	1.494	525 (395)	2.366 (0.687)
2:5-Diformylfuran	310 (224)	1.489 (0.804)	550 (430)	2.278 (1.122)
Furfuraldehyde	317 (257)	1.826 (0.609)	410 (525)	0.071 (0.022)
5-Hydroxymethylfurfuraldehyde	323 (258)	1.472 (0.324)	545 (400)	0.090 (0.067)
Glucuronolactone	302	0.881	525 (395)	1.037 (0.258)
Galacturonic acid	308	0.687	525 (395)	0.715 (0.211)

Reaction at 60°. The absorption curves (Fig. 2) of formylfuroic acid heated in sulphuric acid at 60° (Dische, 1950) were almost exactly the same shape as the corresponding curves for galacturonic acid and the glucuronic acid-containing polysaccharides heparin and oxidized cellulose. The corresponding

curves for 2:5-diformylfuran were considerably different. Glucuronolactone, glucuronic acid and most of the derivatives and compounds of this acid (Table 2) showed little or no absorption, either in the ultraviolet region without added carbazole, or in the visible region with carbazole. As in the reaction at 100°, furfuraldehyde and 5-hydroxymethylfurfuraldehyde showed little colour formation with carbazole; the absorption maxima in the ultraviolet, without added carbazole, were different from those for formylfuroic acid and the uronic acids.

Galacturonic acid (5 mg.), after reaction at 60° and extraction with diethyl ether as described above for glucuronolactone at 100°, showed an absorption spectrum almost identical with that of an ethereal solution of 5-formylfuroic acid. The recovery was similar to that obtained with glucuronolactone at 100°.

DISCUSSION

That 5-formylfuroic acid is the principal light-absorbing product formed from uronic acids in the reactions in sulphuric acid at both 60° and 100° is indicated by (a) the almost identical forms of the absorption spectra of the compounds heated in sulphuric acid in the presence and absence of carbazole, (b) the higher molar absorption intensities shown by formylfuroic acid and (c) the absorption spectrum of a solution of 5-formylfuroic acid in diethyl ether having a maximum at 275 m μ identical in position with those shown by ether extracts of glucuronolactone in the 100° reaction and galacturonic acid in the 60° reaction. The

Fig. 2. Absorption spectra of solutions in sulphuric acid, under the conditions of the carbazole reaction at 60°, with (350–650 m μ) and without (220–350 m μ) added ethanolic carbazole. Each solution contained 5.4 ml. of sulphuric acid and 0.4 ml. of an aqueous solution of one of the substances listed below; the added carbazole was 0.2 ml. of a 0.3% ethanolic solution. A, 2:5-Diformylfuran (100 μ g.); B, 5-formylfuroic acid (100 μ g.); C, D-galacturonic acid (100 μ g.); D, heparin (20 i.u.). Curve A, with carbazole, had $E_{\max}$ 3.2.

Table 2. Intensities of absorption of light, and wavelength maxima, of substances in sulphuric acid in the reaction at 60°, with and without added carbazole

Procedural details are described under Methods. Unless otherwise stated, 0.715 μ mole of each substance was tested. Figures in parentheses refer to secondary absorption maxima. Figures in bold type refer to very low absorption intensities and are approximate.

Substance	Without carbazole, in the range 220–350 m μ		With carbazole, in the range 360–650 m μ	
	$\lambda_{\max}$	$E_{\max}$	$\lambda_{\max}$	$E_{\max}$
5-Formylfuroic acid	295	2.124	535 (405)	1.985 (0.773)
2:5-Diformylfuran	307 (222)	2.062 (1.120)	550 (440)	2.785 (1.250)
Furfuraldehyde	317 (256)	2.391 (0.763)	415 (525)	0.072 (0.011)
5-Hydroxymethylfurfuraldehyde	321 (258)	2.481 (0.555)	425 (537)	0.212 (0.203)
Galacturonic acid	296	0.548	532 (405)	0.568 (0.243)
Glucuronolactone	298	0.026	530	0.009
Glucuronic acid	298	0.029	530	0.011
Menthol glucuronoside	—	—	530	0.008
Methyl β -D-glucofuranuronolactone	296	0.048	530 (405)	0.050 (0.021)
1:2-O-isoPropylidene- α -D-glucofuranuronolactone	298	0.024	530	0.011
Xylose	317 (256)	2.330 (0.720)	425 (525)	0.058 (0.010)
Glucose	324 (257)	0.290 (0.068)	430 (550)	0.012 (0.009)
Oxidized cellulose (100 μ g.)	296	0.155	530 (405)	0.104 (0.070)
Chondroitin sulphate (310 μ g.)	296	0.058	540	0.039
Heparin (20 i.u.)	297	0.141	530 (405)	0.142 (0.067)

formation of formylfuroic acid from uronic acids is analogous, on structural grounds, to the formation of furfuraldehyde from pentoses, of 5-hydroxymethylfurfuraldehyde from hexoses, and of furan-2:5-dicarboxylic acid from the saccharic acids during treatment of the appropriate compound with a strong acid (for a review see Newth, 1951). Decarboxylation, which occurs when uronic acids are boiled with hydrochloric acid (Lefevre & Tollens, 1907) and with 25–27% sulphuric acid (Meller, 1954), may be responsible, in part, for the difference between the absorption curves for formylfuroic acid and the uronic acids in the ultraviolet region in the reaction at 100° without added carbazole. The principal absorption maximum for glucuronolactone and for galacturonic acid is between those for formylfuroic acid and furfuraldehyde, which is one of the products of the decarboxylation reaction. Under the milder conditions of the reaction at 60° it appears probable that formylfuroic acid is the only product of the reaction of uronic acids with sulphuric acid which absorbs light in the ultraviolet region. It is therefore probably the only furan derivative formed in any appreciable quantity. The E values at $\lambda_{\max}$ suggest that conversion of galacturonic acid into formylfuroic acid in the reaction conducted at 60° is 25.8%. If it is assumed that formylfuroic acid is the only material (among the products of the reaction at 100°) that absorbs light in the ultraviolet region, conversion of glucuronolactone into formylfuroic acid is 59%, and of galacturonic acid 46%. It is probable, however, that at 100° some furfuraldehyde is also formed.

The amount of glucuronic acid in 100 μ g. of oxidized cellulose or heparin is certainly considerably less than 0.715 μ mole (for estimates of the actual content, by using the carbazole reaction at 100°, see Bowness, 1957), yet both of these compounds gave a much stronger reaction at 60° than does the equivalent amount of glucuronic acid or of the derivatives listed in Table 2. The absorption curves obtained with these two polysaccharides differed little from those of formylfuroic acid and galacturonic acid. It appears that some types of

linkage (e.g. β -1:4 as in oxidized cellulose and to a lesser extent in the relatively unstable methyl β -glucufuranolactone) but not others (e.g. α -1:4 as in glucuronosides and 1:2-monoacetone glucufuranolactone) can increase the reactivity of glucuronic acid in the reaction at 60°.

SUMMARY

1. The absorption spectra in sulphuric acid, with and without carbazole, of 5-formylfuroic acid, 2:5-diformylfuran, furfuraldehyde, 5-hydroxymethylfurfuraldehyde, furan-2:5-dicarboxylic acid and the uronic acids indicate that 5-formylfuroic acid is responsible for colour formation in the carbazole procedures for estimating uronic acids.

2. 5-Formylfuroic acid appears to be the principal material absorbing ultraviolet light formed under the conditions examined.

3. 2:5-Diformylfuran also gives a colour, with a different absorption spectrum, under the same conditions.

4. Glucuronic acid in oxidized cellulose and heparin gives 5-formylfuroic acid more readily than the uncombined material.

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