757. *The Detection and Differentiation of* 3 : 4- *and* 2 : 5-*Dihydroxyphenyl Compounds Related to Tyrosine.*

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The behaviour of dihydroxyphenyl compounds towards a variety of reagents has been examined. Methods are described by which the 3 : 4- and 2 : 5-dihydroxyphenyl compounds related to tyrosine metabolism can be distinguished.

Two routes for the metabolism of tyrosine lead to dihydroxyphenyl compounds. The action of tyrosinase gives 3 : 4-dihydroxyphenylalanine which is the precursor both of adrenaline-type compounds and of indole derivatives such as adrenochrome. By simple transformations of well-known metabolic occurrence the corresponding 3 : 4-dihydroxyphenyl-ethylamine, -pyruvic acid, and -acetic acid can be derived. The major pathway for tyrosine breakdown, however, has been shown (Knox and LeMay-Knox, *Biochem. J.*, 1951, **49**, 686) to proceed *via* 2 : 5-dihydroxyphenylpyruvic acid and 2 : 5-dihydroxyphenylacetic (homogentisic) acid. The corresponding 2 : 5-dihydroxyphenylacetic acid and 2 : 5-dihydroxyphenylethylamine are therefore of possible occurrence. Both 3 : 4- and 2 : 5-dihydroxyphenyl compounds are strongly reducing and cannot be distinguished by a reagent such as ammoniacal silver nitrate, which in all cases is immediately reduced in the cold. Methods for the differentiation of the 3 : 4- and 2 : 5-dihydroxyphenyl compounds related to tyrosine have therefore been developed, and extended to some analogous compounds.

Previous interest in the detection of dihydroxyphenyl compounds on chromatograms has largely centred on adrenaline and noradrenaline, and James's ferricyanide reagent (James, *Nature*, 1948, **161**, 851; James and Kilbey, *ibid.*, 1950, **166**, 67) has been widely employed with good results (*e.g.*, Hamberg and von Euler, *Acta Chem. Scand.*, 1950, **4**, 1185; Crawford, *Biochem. J.*, 1951, **48**, 203). Although this reagent reacted readily with our compounds, differentiation of the 3 : 4- and the 2 : 5-series was not achieved. The colours obtained are listed in the Table, as are the colours obtained when the treated chromatograms were observed under ultra-violet light which has passed through a Wood's glass filter. Attention was therefore directed to reactions used to detect phenols.

The application of the Folin–Denis reagent (a mixture of phosphomolybdic and phosphotungstic acids) to paper chromatograms has already been described (Cornforth, Dalgliesh, and Neuberger, *Biochem. J.*, 1951, **48**, 598). The reagent has now been found to be much improved by the use of ethanolic ammonia in place of aqueous sodium carbonate for the alkaline spray. The use of phosphomolybdic acid in the estimation of tyrosine metabolites has been developed by Neuberger (*Biochem. J.*, 1947, **41**, 431) and as a detecting reagent in chromatography by Riley (*J. Amer. Chem. Soc.*, 1950, **72**, 5782). Riley concluded that di- and tri-hydric phenols containing *o*- and *p*-hydroxy-groups give immediate blue spots which darken on exposure to ammonia vapour, whereas simple phenols and *m*-polyhydric phenols give no blue colour until after treatment with ammonia. The range of substances tested was small for such wide conclusions. By use of the Folin–Denis reagent, results more useful for the present purpose have been obtained. It has been found that the 2 : 5-compounds all give a blue colour immediately on spraying with the phosphomolybdate–phosphotungstate reagent whereas the 3 : 4-compounds give a marked blue colour only when the paper is made alkaline.

By using only phosphotungstic acid in the reagent, dihydric can be distinguished from monohydric phenols. No colours appear until the paper is made alkaline, whereupon dihydric phenols give spots on a white background, those from the 2 : 5-series being blue and from the 3 : 4-series blue-green. In low concentrations the colours are difficult to distinguish, but the test provides valuable confirmatory evidence. It was long ago shown by Folin (*e.g.*, Folin *et al.*, *J. Biol. Chem.*, 1912, **12**, 239; 1913, **13**, 477) that phosphotungstic acid reacted with dihydric but not monohydric phenols, and the reagent is referred to as Folin's reagent. It also reacts, as is to be expected, with substances such as uric

acid to give blue spots. In the case of the phenolic substances the colour appears rapidly; with uric acid a delay of an hour or hours occurs.

The following abbreviations are used: Dhp = dihydroxyphenyl; Bl = blue; Br = brown; G = green; M = magenta; O = orange; Pk = pink; Pu = purple; Sl = slate-coloured; V = violet; Y = yellow. A dash represents no or negligible colour; p = pale.

		James' ferricyanide		Folin–Denis			Millon's		
	Ninhydrin	visible light	U.V. light	before basification	after basification	Folin's	before heating	after heating	Pauly's
3 : 4-Dhp-alanine	Pu	M	p Pu or Sl	*	Bl	Bl-G	Y	Br	M
3 : 4-Dhp-ethylamine	p Pu	p V	,,	*	Bl	Bl-G	Y	Br	M
3 : 4-Dhp-pyruvic acid	—	p V	,,	p G	Br or Bz-Bl	Bl-G	Y-Br	Br	Br
3 : 4-Dhp-acetic acid	—	p V	,,	*	Bl	Bl-G	Y	Br	Pk
2 : 5-Dhp-alanine	Pu	p V	,,	Bl	Bl	Bl	—	Br	Br-Pk
2 : 5-Dhp-ethylamine	p Pu	p V	,,	Bl	Bl	Bl	—	Br	G
2 : 5-Dhp-pyruvic acid	—	p V	,,	Bl	Bl	Bl	—	Br	G
2 : 5-Dhp-acetic acid	—	p V	,,	Bl	Bl	Bl	—	Br	Br-Pk
2 : 3-Dhp-alanine	Pu	M		*	Bl	G	p Br	Br	O
3 : 4-Dihydroxy-2-methylphenylalanine	Pu	Pk	,,	*	Bl	p Bl	p Y	p Y	M
3 : 4-Dihydroxy-5-methylphenylalanine	Bl-Pu	V	,,	*	Bl	p Bl	p Y	Y	Bl-M
Adrenaline	—	Pk	Y	*	Bl	Bl	p Y	Y-Br	Pk
Noradrenaline	—	M	G	*	Bl	Bl	p Y	Br	Pk

* A pale blue colour may appear before the alkaline spray, but this increases very markedly on basification.

Millon's reaction has been examined both on paper and in the test-tube. On paper satisfactory results have been obtained by using diluted bench reagent. Compounds of the 3 : 4-series give a yellow colour immediately on spraying. When the air-dried paper is heated at 100° for a short while, the 2 : 5-compounds appear as brown spots and the initially yellow spots of the 3 : 4-series also become brown. The procedure renders the paper fragile, but not unduly so. In the test tube Medes's modification (*Biochem. J.*, 1932, **26**, 917) of the method of Folin and Ciocalteau (*J. Biol. Chem.*, 1927, **73**, 627) was used. All the dihydroxyphenyl compounds give copious coloured precipitates when heated with the mercuric sulphate reagent, but give no colour on centrifugation and treatment with nitrite. On the other hand both *N*-methyltyrosine and *p*-hydroxyphenylpyruvic acid behave like tyrosine itself (cf. Neuberger, *loc. cit.*; Knox and LeMay-Knox, *loc. cit.*). Neither 3 : 4- nor 2 : 5-dihydroxyphenyl compounds would therefore interfere with estimations by this procedure.

Pauly's reagent (diazotised sulphanilic acid) was found to give valuable information. The 3 : 4-series, except for the pyruvic acid, all give an immediate pink or magenta colour, whereas the 2 : 5-series give a yellow colour becoming a pinkish-brown or green. These colours can vary under different conditions, and reference compounds should therefore be used.

The Folin–Denis, Folin, and Pauly reagents are all of high sensitivity for the detection of dihydroxyphenyl compounds, and together enable the 3 : 4- and the 2 : 5-series to be differentiated.

Experimental

Chromatography.—Chromatograms were run on Whatman No. 4 paper by the descending technique, with the organic phase of butanol–acetic acid–water mixtures of varying compositions as solvent (cf. preceding paper). 3 : 4- and 2 : 5-Dihydroxyphenylpyruvic acid did not run satisfactorily in this solvent and the results recorded were obtained with spots of a solution applied to filter paper and allowed to dry.

Materials.—3 : 4- and 2 : 5-Dihydroxyphenyl-alanines, -ethylamines, -pyruvic acids, and -acetic acids were supplied by Dr. A. Neuberger, and 2 : 3- and 3 : 4-dihydroxy-2- and -5-methylphenylalanine by Mr. J. Harley-Mason. The adrenaline and noradrenaline were from commercial sources.

Reagents.—(1) *Folin–Denis reagent.* This was used as described by Cornforth *et al.* (*loc. cit.*) with the exception that the alkaline spray was made by mixing 1 volume of aqueous ammonia (*d* 0·88) with 2 volumes of ethanol. This gives rise to very much less background interference than is obtained with a sodium carbonate spray.

(2) *Folin's reagent.* The method for making Folin–Denis reagent described in the Merck Index (fifth edn., Merck, Rahway, N.J., p. 725) was followed, with the omission of phosphomolybdic acid. The resultant reagent had a very pale blue tint in contrast to the pale green of the Folin–Denis reagent. As with the latter it is important to use an all-glass spray. After being sprayed with the reagent and allowed to dry the paper was sprayed with the ethanolic ammonia solution described above.

(3) *Millon's reagent.* For spraying on chromatograms the bench reagent prepared as described in the Merck Index (*op. cit.*, p. 839) was diluted with 2 volumes of water.

(4) *Pauly's reagent.* Diazotised sulphanilic acid was diluted with an equal volume of ethanol before spraying (cf. Evans, Parr, and Evans, *Nature*, **164**, 674) and when dry the chromatograms were made alkaline with the ethanolic ammonia solution described above.

Results.—The colours obtained with the different reagents are summarised in the accompanying Table. For examining chromatograms under ultra-violet light a lamp with a Wood's glass filter was used.

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