Synthesis and Resolution of 2:5-Dihydroxyphenylalanine

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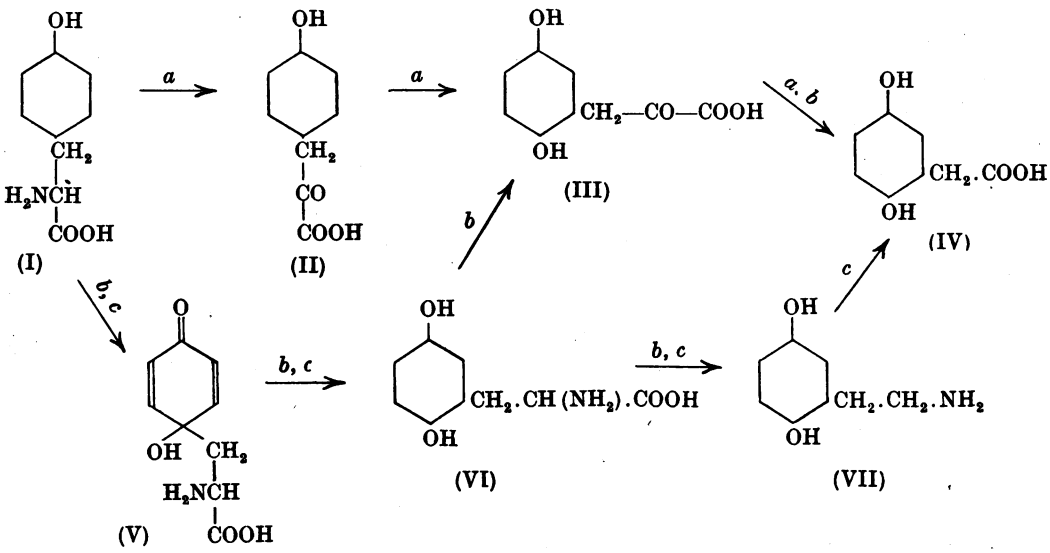
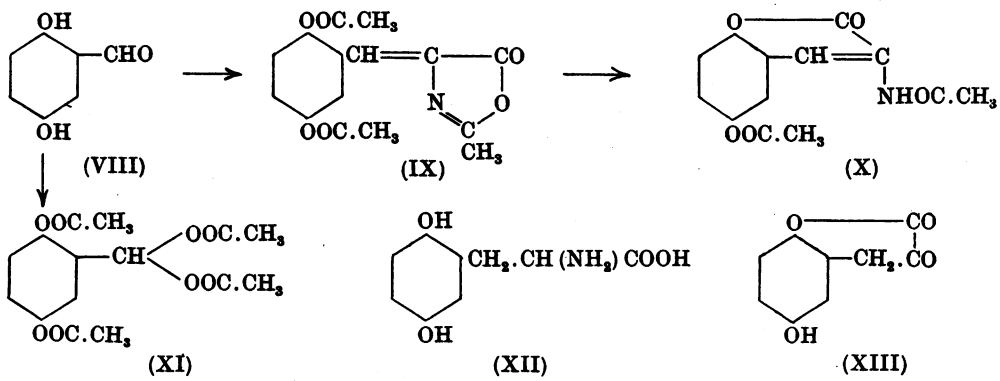
It is generally believed that the main pathway of the metabolism of the amino-acids phenylalanine and tyrosine in mammals involves the intermediate formation of homogentisic acid. In alcaptonuric man, and in various laboratory animals reared under certain dietary conditions, the amount of homogentisic acid excreted in the urine is proportional to the intake of the two aromatic amino-acids; this indicates that the oxidation of tyrosine to a quinol with an accompanying shift of the side chain can be performed by mammals. Moreover, the recent isolation by Fishberg (1948), in cases of enterogenous cyanosis, of 1:4-benzoquinone-2-acetic acid, the quinone corresponding to homogentisic acid, has demonstrated that, even in man, the formation of compounds related to homogentisic acid is not confined to alcaptonurics and premature babies. But the assumption that such a reaction represents the main normal pathway of metabolism for these aromatic amino-acids rests on an argument by analogy, and no conclusive evidence for this hypothesis has as yet been adduced. Even in alcaptonuria the exact mechanism of the conversion of tyrosine to homogentisic acid is still obscure. The oxidation of a phenol to a quinol is a fairly common biological reaction; thus small amounts of quinol are excreted on ingestion of large doses of phenol by the dog (Baumann & Preusse, 1879) and salicylic acid gives rise in man (Baldoni, 1908) and in the rat (Lutwak-Mann, 1943) to gentisic acid. But no exact biological analogy exists for the migration of a substituent originally occupying the para position. The formation of thyroxine from diiodotyrosine which occurs so readily *in vivo* and *in vitro* must involve a somewhat similar mechanism (see Pitt-Rivers, 1948). However, in the latter case, the side chain is eliminated whilst with tyrosine it is shifted to the adjacent position in the ring. It is generally assumed that tyrosine (I) is first converted to the corresponding keto acid (II) which is then further oxidized in the ring to give 2:5-dihydroxyphenylpyruvic acid (III) which on oxidative decarboxylation would yield homogentisic acid (IV). But the fact that *p*-hydroxyphenylpyruvic acid is far less effective than tyrosine as a precursor of homogentisic acid in the alcaptonuric (Neubauer, 1909; Fromherz & Hermanns, 1914) suggests that oxidation of the ring precedes that of the side chain. The small amount of extra homogentisic acid excreted on administration of *p*-hydroxyphenylpyruvic acid may be ascribed to its reconversion into tyrosine by amination. As Neubauer (1928) points out, at least two other possibilities (*b*, *c*) must be considered.



Thus a second hydroxyl group might be introduced into tyrosine to give the dienone (V) which would rearrange to 2:5-dihydroxyphenylalanine (VI); this could then be oxidized through the keto acid to homogentisic acid (mechanism *b*) or be first decarboxylated to the amine (VII) and then oxidized (mechanism *c*). In order to test these possibilities it was decided to prepare and resolve 2:5-dihydroxyphenylalanine. The synthesis of 2:5-dihydroxyphenylethylamine, another possible intermediate, is described in the accompanying paper (Leaf & Neuberger, 1948).

Reaction of gentisic aldehyde with acetylglycine

The racemic amino-acid was prepared in two ways. In the first synthesis gentisic aldehyde (VIII) was condensed with acetylglycine in the presence of acetic anhydride and sodium acetate. From the crude product three substances were isolated by fractional crystallization. The first compound had m.p. 140–141° and crystallized in yellow needles; it had one fairly stable and two very labile acetyl groups. This fact, the colour and elementary analysis indicate that this is the acetylated azlactone (IX). The second substance of m.p. 226–227° is colourless and contains one labile and one stable acetyl group. It is assumed to be 3-acetamido-2-keto-6-acetoxycoumarin (X) or the tautomeric 3-acetimido-2-keto-6-acetoxychroman. Several workers have obtained both the benzamidocoumarin and the benzoylated azlactone in the condensation of salicylaldehyde and hippuric acid (Rebuffat, 1885; Plöchl & Wolfrum, 1885; Erlenmeyer & Stadlin, 1904), and similar results have been obtained in the condensation of 2:4-dihydroxybenzaldehyde and hippuric acid (Deulofeu, 1936). Dakin (1929), on the other hand, isolated only an acetylated azlactone from the condensation of salicylaldehyde and acetylglycine, and similarly no coumarin derivative was isolated in the reaction of gentisic aldehyde with hippuric acid (Neubauer & Flatow, 1907). The separation of these two products is troublesome, and attempts were, therefore, made to establish conditions under which only the azlactone would be formed. It is generally accepted that the first step in the azlactone synthesis is the formation of 2-phenyl- or 2-methyloxazolone, the reactive methylene group of which then reacts with the aldehyde (see Carter, 1946). Two geometrically isomeric azlactones will generally result, and only one of these will contain the reactive carbonyl group and the phenolic ring suitably placed for esterification to proceed. Coumarin formation thus appeared to be a secondary reaction which might be facilitated by the presence of a free hydroxyl group and by prolonged heating. It was hoped to eliminate formation of the coumarin by using 2-acetoxybenzaldehydes. However, this aim was not fully achieved. Acetoxybenzaldehydes can be readily prepared in almost quantitative yield by treating the hydroxyaldehydes with pyridine and acetic anhydride; the products are distillable crystalline substances. Condensation of 2-acetoxybenzaldehyde with hippuric acid and of 2:5-diacetoxybenzaldehyde with acetylglycine yielded mainly, but not exclusively, azlactone if the period of heating was short (30 min.). If heating was continued for 1–2 hr., larger amounts of coumarin were obtained. The conclusion that even the acetylated azlactone is converted to the coumarin under the conditions of the condensation was supported by the observation that heating pure acetylated azlactone of m.p. 140° with acetic anhydride and sodium acetate at 100° produced appreciable amounts of coumarin.



The third product of the reaction was nitrogen-free, and from elementary analysis and acetyl content was identified as 2:5-acetoxybenzylidene diacetate (XI). The formation of diacyl derivatives of aldehydes in the preparation of azlactones, has not, as far as we are aware, hitherto been reported, and is of particular interest in view of the suggestion of Erlenmeyer & Früstück (1895) that such diacetates are intermediates in the formation of azlactones. It has been found, however, that the diacetates of benzaldehyde, 2-acetoxy- or 2:5-diacetoxybenzaldehyde do not form azlactones under the appropriate conditions. It seems more probable that the formation of such acyl derivatives removes aldehydes from the reaction, and explains the low yields of azlactones observed in some cases, e.g. when acetylglycine is used.

Both the coumarin (X) and the azlactone (IX) yield on hydrolysis with dilute HCl 6-hydroxy-2: 3-diketochroman (XIII) which on treatment with alkali, in the absence of O_2, followed by careful acidification gives 2:5-dihydroxyphenylpyruvic acid (III). Both these compounds have already been obtained by Neubauer & Flatow (1907) by a less convenient method. On treatment with red phosphorus and HI the amino-acid (XII) is obtained in good yield from either (IX) or (X). An aqueous solution of DL-2:5-dihydroxyphenylalanine is readily oxidized in air at a

slightly alkaline pH and slowly even at neutrality giving a melanin-like pigment. Hirai (1927) claimed to have prepared the racemic amino-acid by condensation of 2:5-dimethoxybenzaldehyde with glycine anhydride followed by reduction and hydrolysis. The properties of the compound obtained by the Japanese worker differ greatly, however, from those of the product described in this paper. Thus, Hirai gives a m.p. of 203–204°, whilst we found a m.p. of 235°. The amino-acid obtained by us crystallizes with 1 mol. of water which is not removed by drying at 15 mm. pressure at room temperature over P_2O_5. On prolonged drying at 110° the water is lost, but the anhydrous material hydrates again quickly. This property is not mentioned by Hirai. Moreover, Hirai's compound was apparently easily soluble in water, whilst we found the solubility of the racemic amino-acid to be less than 1% at 18° in water. No suggestion can be offered as to the cause of this discrepancy.

Resolution of 2:5-dihydroxyphenylalanine

In the course of the work it was found that *N*-acyl derivatives of 2:5-dihydroxyphenylalanine lactonize readily, are easily oxidized, and are, therefore, not convenient for resolution. It was decided to resolve instead a suitable derivative of 2:5-dimethoxyphenylalanine and to remove the methyl groups afterwards. The 2:5-dimethoxybenzaldehyde required for this synthesis was made both by the Reimer-Tiemann method from 4-methoxyphenol followed by methylation with methyl sulphate (cf. Tiemann & Müller, 1881), and from 1:4-dimethoxybenzene by the modification of the Gattermann synthesis introduced by Adams & Levine (1923). Gulland & Virden (1928*a*, *b*) used a somewhat similar method for the preparation of this aldehyde. The 2-benzamido-3-(2′:5′-dimethoxyphenyl)-acrylic acid obtained from the azlactone, prepared by the condensation of the aldehyde with hippuric acid, was rather resistant to hydrogenation with a palladium or platinum catalyst (cf. Waser, 1925), but the sodium salt was readily reduced under pressure by H_2 and Raney's nickel. Attempts to resolve the resulting 2-benzamido-3-(2′:5′-dimethoxyphenyl)-propionic acid with brucine or quinine were unsuccessful, but (+)- or (−)-α-phenylethylamine respectively yielded two pairs of diastereoisomeric salts which could be separated by fractional crystallization from water. Short hydrolysis with hydriodic acid yielded the active amino-acids without any appreciable racemization. The optically active amino-acid resembles the racemic compound in most of its properties; thus it also crystallized with 1 mol. of water which is difficult to remove. However, the active amino-acid is more insoluble in cold water than the racemic compound and can be conveniently recrystallized from that solvent. D- and L-Configurations were assigned to the dextro- and laevo-rotatory amino-acids respectively on the basis of the following considerations discussed in detail elsewhere (Neuberger, 1948).

(1) All D-amino-acids show a decrease in dextro- or increase in laevo-rotation when the neutral molecule is transformed into the corresponding cation, i.e. on addition of acid. The rotation of (+)-2:5-dihydroxyphenylalanine in water cannot be measured accurately due to low solubility. However, an $[\alpha]_D$ of $+40° \pm 3$ was obtained by using a 4 dm. tube. In N-HCl this value is decreased to +7·8. The difference in molecular rotation, which is about 69 and of the same order as that found for other α-amino-acids containing β-aryl groups, indicates that the (−)-acid has L-configuration. Moreover, L-3:4-dihydroxyphenylalanine (Waser & Lewandowski, 1921) for which the L-configuration is established by direct transformation from L-tyrosine has rotations similar, both in water and in acid, to those of the 2:5 compound.

(2) Another empirical and apparently generally applicable rule is that the hydantoins of L-amino-acids have rotations which are negative and relatively large. The $[\alpha]_D$ of the hydantoin of (−)-2:5-dihydroxyphenylalanine was found to be −124·5° in aqueous ethanol.

(3) An enzyme present in guinea-pig kidney decarboxylates the (−)-amino-acid quantitatively (Blaschko, Holton & Sloane Stanley, 1948) whilst the dextro-rotatory substance remains unchanged. This enzyme is identical with, or closely similar to, dopa decarboxylase which is specific for L-3:4-dihydroxyphenylalanine (Blaschko, 1942–3). Since both mammalian and bacterial decarboxylases act on L-amino-acids only, the enzymic decarboxylation of the (−)-amino-acid supports strongly the conclusions drawn from the physical data, and there can be no doubt that the laevorotatory 2:5-dihydroxyphenylalanine has L-configuration.

The biological behaviour of 2:5-dihydroxyphenylalanine

The L-amino-acid, if given by mouth to rats in daily doses up to 1 g., is apparently readily metabolized; no reducing substances could be detected in the urine, even after acid hydrolysis. The D-isomeride, too, can apparently be oxidized completely by the rat, if the dose does not exceed 200 mg./100 g. body weight. Larger doses cause the excretion of small amounts of reducing material which, from its solubility, appears to be the unchanged amino-acid. In normal man also, 5 g. of the L-amino-acid were completely metabolized, whilst the same quantity of the D-compound gave rise to the appearance of small amounts of a reducing substance in the urine. The amino-acid appears to be completely non-toxic on oral, subcutaneous and intraperitoneal administration to rabbits, mice, rats and guinea pigs. Experiments with the racemic compound already reported (Neuberger, Rimington & Wilson, 1947) indicate that it is, at least partially, converted into homogentisic acid by the alcaptonuric. Experiments with the active amino-acids will be carried out, when an opportunity arises. The findings of Blaschko *et al.* (1948) that the L-amino-acid can be decarboxylated by a mammalian enzyme have already been mentioned. The resulting 2:5-dihydroxyphenylethylamine is also readily metabolized by man and by the rat (Leaf & Neuberger, 1948). On the basis of these findings, it appears probable that the normal metabolism of tyrosine goes at least partly through the stages 2:5-dihydroxyphenylalanine and 2:5-dihydroxyphenylethylamine to homogentisic acid (cf. path *c* on p. 599). The alternative that the quinolic amino-acid is first oxidized to the keto acid and then decarboxylated (path *b*

on p. 599) is less likely, since 2:5-dihydroxyphenylpyruvic acid like *p*-hydroxyphenylpyruvic acid is not a very effective precursor of homogentisic acid (Neubauer & Flatow, 1907). However, the facts presented here only indicate that the suggested pathway is possible and in accordance with known facts. The hypothesis will have to be substantiated by further experiments with isolated enzymes or tissue slices.

EXPERIMENTAL

*Preparation of compounds**

Reaction of gentisic aldehyde with acetylglycine. 13·8 g. of gentisic aldehyde (Neubauer & Flatow, 1907), acetylglycine (11·7 g.), anhydrous sodium acetate (9 g.) and acetic anhydride (40 ml.) were intimately mixed and refluxed for 2–3 min. The mixture was then heated for a further 1 hr. on a water bath. The partly crystalline mixture was cooled, the solid broken up and triturated with ice water. The solid was filtered off and washed with ice water, ethanol (40 ml.) and ether. The combined mother liquors were extracted with ethyl acetate, and the ethyl acetate extracts dried and concentrated. A further crop of solid material was thus obtained. Fractional crystallization from ethanol and benzene yielded three substances: (*a*) A compound of m.p. 226–227°, crystallizing in needles, almost colourless and almost insoluble in cold ethanol. This substance was identified as 3-*acetamido*-2-*keto*-6-*acetoxycoumarin*. (Found: C, 59·4; H, 4·2; N, 5·5. $C_{13}H_{11}O_5N$ requires: C, 59·8; H, 4·2; N, 5·36%.) Labile acetyl groups were estimated as follows: 0·261 g. of the material was dissolved in 25 ml. 0·1 N-NaOH and allowed to stand at room temperature for 2 hr.; 25 ml. 0·1 N-HCl were then added and the mixture was steam-distilled. The distillate required 10·2 ml. 0·1 N-NaOH for neutralization to cresol red; this gives a labile acetyl content of 16·8%. $C_{13}H_{11}O_5N$ requires a labile acetyl content of 16·4%. Total acetyl content was estimated as follows: 0·261 g. of the material was refluxed for 4 hr. with 20 ml. of 3 N-H_2SO_4 and then steam-distilled. On titrating the distillate with 0·1 N-NaOH, using cresol red as indicator, 20·5 ml. were required, hence total acetyl content = 33·8% and by difference stable acetyl content = 17·0%. $C_{13}H_{11}O_5N$ requires 16·4%. The analytical results and the lack of colour leave no doubt that the structure assigned to this compound is correct. (*b*) The second compound was more soluble in ethanol and fairly soluble in hot benzene. It crystallized in small bright yellow needles and had m.p. 140–141°. It was identified as 2-*methyl*-4-(2′:5′-*diacetoxybenzylidene*)-*oxazolone*. (Found: C, 58·9; H, 4·55; N, 4·7. $C_{15}H_{13}O_6N$ requires: C, 59·4; H, 4·3; N, 4·6%.) Labile and stable acetyl groups were estimated as described above. (Found: labile acetyl, 28·8; stable acetyl, 14·4. $C_{15}H_{13}O_6N$ requires labile acetyl, 28·4; stable acetyl, 12·2%.) (*c*) The third compound was more soluble in ethanol and benzene than the other two. It was colourless, crystallized in tablets of m.p. 127 and did not contain N. It was identified as 2:5-*diacetoxy-benzylidene diacetate*. (Found: C, 55·7; H, 5·14. $C_{15}H_{16}O_8$ requires: C, 55·55; H, 4·9%.) Mixed m.p. with a sample prepared by an independent method (see below) was 127°. The crystalline products were isolated in a yield of altogether 35–40%. The crude material consisted largely of the azlactone and was obtained in a yield of about 50%.

* All melting points are uncorrected.

Preparation of aldehyde diacetates and acetoxyaldehydes

2-*Acetoxybenzaldehyde*. Salicylaldehyde (18·3 g.) was added to dry pyridine (40 ml.); the solution was cooled to −10° and acetic anhydride (15 g.) added over 5 min. The mixture was left for 2 hr. and then poured into ice water. An oil precipitated which soon crystallized. The material was filtered off, washed with ice water and dried. Yield was 90% of the theoretical. The solid was distilled at 142° (15 mm.), m.p. was 36–37°. Perkin (1868) gives m.p. 37°.

2:5-*Diacetoxybenzaldehyde*. 2:5-Dihydroxybenzaldehyde (6·58 g.) was acetylated with pyridine (15 ml.) and acetic anhydride (9·5 ml.) as described above. The material, which crystallized on pouring the solution into water, was filtered off and dried. Yield of 2:5-*diacetoxybenzaldehyde* was 95%. 10 g. were recrystallized from ligroin (200 ml.) and had m.p. 68°; on further recrystallization the m.p. was raised to 68·5–69°. (Found: C, 59·2; H, 4·7. $C_{11}H_{10}O_5$ requires: C, 59·4; H, 4·5%.)

2:5-*Diacetoxybenzylidene diacetate*. 2:5-Diacetoxybenzaldehyde (2·2 g.) was dissolved in acetic anhydride (5 ml.); the mixture was cooled to −5° and conc. H_2SO_4 (0·05 ml.) was added. Crystals appeared almost at once. After 1 hr. at 0° the mixture was poured into water containing $NaHCO_3$ (15 g.) and allowed to stand at 0° for 1 hr. The crystals were then filtered off and dried. Recrystallized from ethanol, the substance had m.p. 128·5°. Yield was 90% of the theoretical. The diacetates of benzaldehyde and salicylaldehyde were prepared in a similar manner and were found to have the correct m.p.'s.

Both benzylidene diacetate and 2-acetoxybenzylidene diacetate were heated for 1 hr. on a water bath with 1 equiv. of hippuric acid, 1 equiv. of anhydrous sodium acetate and 4 equiv. of acetic anhydride. No azlactones could be isolated from these mixtures.

Azlactone formation with acetoxyaldehydes. (*a*) 2-Acetoxybenzaldehyde and hippuric acid. A mixture of 2-acetoxybenzaldehyde (25·7 g.), hippuric acid (28 g.), anhydrous sodium acetate (12·7 g.) and acetic anhydride (60 ml.) was heated on the water bath for 20 min. The solution was cooled, poured into ice water containing ethanol (50 ml.). The crystalline solid was filtered off and dried. It weighed 50 g. Fractional crystallization by the method of Asahina (1930) gave 4·5 g. of pure coumarin, whilst the rest of the material consisted almost entirely of the azlactone. A longer period of heating or use of salicylaldehyde instead of the acetyl derivative decreased the crude yield and increased the proportion of the coumarin. (*b*) 2:5-Diacetoxybenzaldehyde and acetylglycine. A mixture of 2:5-diacetoxybenzaldehyde (11·1 g.), acetylglycine (5·8 g.), sodium acetate (4·5 g.) and acetic anhydride (25 ml.) was refluxed for 10 min. and then heated on the water bath for a further 0·5 hr. Working up as described above gave a 60% yield of the acetylated azlactone contaminated with some coumarin. The yield was slightly better, and the product less heterogeneous than that prepared from 2;5-dihydroxybenzaldehyde.

Preparation of DL-2:5-*dihydroxyphenylalanine and* 2:5-*dihydroxyphenylpyruvic acid*

2:5-*Dihydroxyphenylpyruvic acid*. The keto acid and the amino-acid have been prepared from the corresponding 2-methyloxazolone, the coumarin or the crude mixture.

Yields from the pure compounds were higher. Only the hydrolysis of the azlactone needs to be described in detail: 5 g. of the 2-methyloxazolone were refluxed with a mixture of equal parts 3 N-HCl and glacial acetic acid for 5 hr. The solution was concentrated *in vacuo*, and the lactone of the keto acid was obtained in a yield of 80%. After recrystallization from water and treatment with charcoal the m.p. of the now colourless crystals was 225°. Neubauer & Flatow (1907) state that the m.p. is above 220° and not sharp. The lactone was converted to the free keto acid as described by these authors. It had all the properties described by them.

DL-2:5-*Dihydroxyphenylalanine.* 2-Methyl-4-(2′:5′- diacetoxybenzylidene) oxazolone (10 g.) was refluxed with a mixture of glacial acetic acid (60 ml.), red phosphorus (2 g.) and 20 ml. HI (sp.gr. 1·7) for 1 hr. The solution was filtered hot and concentrated under reduced pressure to dryness. The residue was taken up in water and extracted with ether. The aqueous solution was again evaporated to dryness and the solid dissolved in water, 20% (w/v). Lead acetate was then added carefully, until the pH was about 1·8–2·0. If the pH is allowed to rise to 3·0 and above, the Pb complex of the amino-acid is precipitated. PbI_2 was then filtered off and the solution treated with H_2S. The precipitate was filtered off, and the solution concentrated *in vacuo* under N_2 to low bulk. On addition of pyridine to pH 5·0 the amino-acid crystallized out. It was recrystallized from water containing a trace of SO_2. Yield of recrystallized material was on the average 50%. A further 10–20% could be recovered from the mother liquors. M.p. was 235°. The substance crystallizes with 1 mol. of water which was not removed by drying over P_2O_5 at room temperature at 10 mm. pressure. On prolonged drying at 110° and at 1 mm., the water is lost. Loss of weight on drying 7·9%; $C_9H_{13}O_5N$ requires 8·4%. The amino-acid was analyzed as the hydrate. (Found: C, 49·9; H, 6·0; N, 6·5. $C_9H_{11}O_4N.H_2O$ requires: C, 50·2; H, 6·1; N, 6·5%.) The substance gave a positive ninhydrin reaction, reduced silver nitrate slowly at pH 4–5 and instantaneously at alkaline reaction and reduced phosphomolybdic acid in acid solution.

Alternative method for the synthesis of DL-2:5-*dihydroxyphenylalanine*

2:5-*Dimethoxybenzaldehyde.* This was prepared by two methods. (*a*) Gattermann synthesis. The method described here is shorter and more convenient than that of Gulland & Virden (1928*a*, *b*). Into a three-necked flask fitted with a mercury-sealed stirrer, a reflux condenser and an inlet tube was placed 1:4-dimethoxybenzene (30 g.), dry benzene (90 ml.) and zinc cyanide (40·4 g.). The mixture was cooled in ice and dry HCl was passed in, whilst the mixture was rapidly stirred, until saturated with HCl. Finely powdered $AlCl_3$ (44 g.) was then added and the temperature raised to 45°. The mixture was kept at that temperature for 3·5 hr., whilst a slow stream of HCl was passed in. The mixture was then poured into 3 N-HCl (500 ml.), refluxed for 0·5 hr. and cooled. Ethyl acetate (200 ml.) was added, the organic layer separated, and the aqueous solution again extracted with ethyl acetate. The combined extracts were then dried, the solvent removed and the remaining oil distilled. A small amount of unchanged dimethoxybenzene came over below 130°, whilst the bulk of the material distilled sharply at 154° (18 mm.). It crystallized in the receiver and had m.p. 53°. Yield was 73% of the theoretical. (*b*) Reimer-Tiemann synthesis. NaOH (80 g.) was dissolved in water (100 ml.). To this solution, which was kept at 65–70°, was added 33 g. of *p*-methoxyphenol (Robinson & Smith, 1926) and chloroform (20 g.). The solution was kept at 70° by alternate cooling and warming. When the reaction had subsided a second lot of chloroform (20 g.) was added, and after 10 min. a third lot. The mixture was then refluxed for 1 hr.; excess chloroform was removed by distillation and the solution acidified with 5 N-H_2SO_4. The aldehyde was then distilled in steam, 1·5–2 l. of distillate being collected. The distillate was extracted with ether and concentrated; the resulting oil was distilled *in vacuo* under N_2. B.p. was 144·5° (20 mm.). The crude aldehyde (21 g.) was methylated with methyl sulphate (1·2 equiv.) and 2 N-NaOH (1·2 equiv.) at 65°. The aldehyde after cooling was filtered off and the mother liquor extracted with ethyl acetate. The crystalline material and the oil from the ethyl acetate extraction were combined and distilled. B.p. was 160° (20 mm.). Overall yield was 35–40%.

2-*Phenyl*-4-(2′:5′-*dimethylbenzylidene*) *oxazolone and* DL-2:5-*dihydroxyphenylalanine.* A mixture of 2:5-dimethoxybenzaldehyde (15 g.), hippuric acid (16·2 g.), anhydrous sodium acetate (7·4 g.) and acetic anhydride (50 ml.) was heated on a water bath for 1·5 hr. The mixture was cooled to 0° and ethanol (50 ml.) added slowly. After standing for 1 hr. at 0° it was poured into ice water (250 ml.) and left at 0° for 2 hr. The crystalline solid was filtered off, washed with water, ethanol and ether. Yield was 75%. M.p. was 169–170°. After recrystallization from benzene m.p. was 172°. Gulland & Virden (1928*b*) give m.p. 170–172°. (Found: C, 69·8; H, 5·00; N, 4·42. Calc. for $C_{18}H_{15}O_4N$: C, 69·9; H, 4·85; N, 4·53%.) The azlactone could be converted directly to DL-2:5-dihydroxyphenylalanine: 40 g. of the azlactone were added to a mixture of glacial acetic acid (160 ml.), red phosphorus (10 g.) and 125 ml. HI (sp.gr. 1·7). The mixture was refluxed for 15 min., the methyl iodide which had formed was distilled off and refluxing continued for another 2 hr. Isolation was carried out as described above. This method is more convenient for the preparation of the amino-acid than the one described above, using acetylglycine. However, it cannot be used for the preparation of the keto acid.

Resolution

2-*Benzamido*-3-(2′:5′-*dimethoxyphenyl*)-*acrylic acid.* This substance has already been described by Gulland & Virden (1928*b*), who found m.p. 195–196°. Such material, though it analyzes correctly, was found on reduction to give an acid which was not satisfactory for resolution, and it was necessary to recrystallize several times before reduction. The azlactone (30·9 g.) was partially dissolved in ethanol (400 ml.) and 0·5 N-NaOH (230 ml.) was then added to the boiling solution which was left at 90° for 20 min. The solution was cooled in ice and acidified with 2 N-HCl (80 ml.). The crystalline precipitate was filtered off and washed with cold water. Yield was 90%. The acid was recrystallized first from 50% (v/v) ethanol, twice from 50% (v/v) acetic acid and finally from chloroform and ligroin. The crude product had m.p. 195° as described by Gulland & Virden (1928*b*), but after repeated recrystallization it was 188°. (Found: C, 65·9; H, 5·1; N, 4·2. Calc. for $C_{18}H_{17}O_5N$:

C, 66·1; H, 5·2; N, 4·3%.) The lowering of m.p. on recrystallization may be associated with the possibility of *cis-trans* isomerism in the acrylic acid.

DL-2-*Benzamido*-3-(2′:5′-*dimethoxyphenyl*)-*propionic acid.* 75 g. of the acrylic acid were dissolved in N-NaOH (250 ml.), Raney-nickel suspension (20 g.) was added and the vol. made up to 1 l. Reduction occurred quickly at an initial pressure of 80 atm. at 15°. When the reduction had stopped the solution was filtered and acidified. Yield was almost quantitative. The *benzamidopropionic acid* was recrystallized from 30% (v/v) acetic acid. It had m.p. 176·5°. (Found: C, 65·5; H, 5·9; N, 4·3. $C_{18}H_{19}O_5N$ requires: C, 65·7; H, 5·8; N, 4·4%.)

(−)-*Phenylethylamine salt of* L-2-*benzamido*-3-(2′:5′-*dimethoxyphenyl*)-*propionic acid.* The racemic acid (49 g.) and (−)-phenylethylamine (18·8 g.) were dissolved in methanol (300 ml.) and the solution evaporated to dryness. The crystalline residue was dissolved in boiling water (1500 ml.) and allowed to cool to 15°. The crystalline solid was filtered off, and again dissolved in water (1500 ml.) and cooled to 15°. A third recrystallization was done from 2 l. of water. Rotation and m.p. had by then become constant. Yield was 90% of the theoretical (31·5 g.). M.p. was 180°; $[\alpha]_D^{18°}$ −13·7° in methanol (*c*, 2·0). The salt crystallized as a *monohydrate.* (Found: C, 66·4; H, 6·8; N, 6·0. $C_{26}H_{30}O_5N_2.H_2O$ requires: C, 66·7; H, 6·8; N, 5·7%.)

(−)-*Phenylethylamine salt of* D-2-*benzamido*-3-(2′:5-*dimethoxyphenyl*)-*propionic acid.* The mother liquors amounting to 5 l. were combined and concentrated under reduced pressure (100 mm.) to about 1 l. Small amounts of crystalline material which separated out during the concentration, and which consisted of mixtures of diastereoisomers, were collected and preserved for a later resolution. The solution was then cooled to 0°, and the crystalline precipitate of m.p. 160° filtered off; a further crop was obtained by concentrating the solution to 300 ml. and cooling to 0°. The combined solids were again recrystallized first from water and then from ethanol. The yield was 24 g. The mother liquors were combined and concentrated. M.p. was 167–168°, $[\alpha]_D^{18°}$ +9·1° in methanol (*c*, 2·0). This salt also crystallized as *monohydrate.* (Found: C, 66·3; H, 6·5; N, 5·9. $C_{26}H_{30}O_5N_2.H_2O$ requires: C, 66·7; H, 6·8; N, 6·0%.)

The two salts of (+)-phenylethylamine were also prepared and had the expected properties: +D-*salt*: m.p. 180°; $[\alpha]_D^{19°}$ +13·8° in methanol (*c*, 2·0); +L-*salt*: m.p. 166°; $[\alpha]_D^{19°}$ −9·0° in methanol (*c*, 2·0). The −D- and +L-salts were probably optically not completely pure, but further recrystallization did not raise the rotation or m.p. any further.

D- *and* L-2-*Benzamido*-3-(2′:5′-*dimethoxyphenyl*)-*propionic acids.* The −L- and +D-salts respectively were decomposed as follows: 45 g. of the salt were dissolved in hot water (1 l.) and 5N-NaOH (50 ml.) was added, whilst the solution was shaken. The phenylethylamine was removed by repeated extraction with chloroform and recovered in the usual manner. The aqueous solution was acidified with 5N-HCl (55 ml.) and the precipitate filtered off and dried. The material was recrystallized first from chloroform-light petroleum (b.p. 120°) and then from 30% (v/v) acetic acid. M.p. of both compounds was 170°. The L-*acid* had $[\alpha]_D^{17°}$ −32·5° in ethanol (*c*, 2·0), whilst the D-*acid* had $[\alpha]_D^{16°}$ +32·8° in ethanol (*c*, 2·0). The L-acid only was analyzed. (Found: C, 65·6; H, 5·9; N, 4·1. $C_{18}H_{19}O_5N$ requires: C, 65·7; H, 5·9; N, 4·25%.) The +L- and −D-salts were decomposed in similar manner. The corresponding D- and L-benzamido acids had m.p. 167 and 168° respectively and the following rotations: D-acid: $[\alpha]_D^{17°}$ +30·5° in ethanol (*c*, 2·0); L-acid: $[\alpha]_D^{17°}$ −31·0° in ethanol (*c*, 2·0).

D- *and* L-2:5-*Dihydroxyphenylalanine.* 10 g. of the active benzoyl compound were hydrolyzed with 80 ml. of a mixture of equal parts of HI (sp.gr. 1·7) and glacial acetic acid which had previously been refluxed for 15 min. with red P (0·4 g.) and filtered. The mixture was refluxed for 15 min., methyl iodide removed by distillation, and the hydrolysis continued for another 1·25 hr. The amino-acids were isolated as described above. Yield of recrystallized amino-acid was 65–70%. The L-*amino-acid* had m.p. 265°, $[\alpha]_D^{17°}$ −8·1° in N-HCl (*c*, 2·0). (Found: C, 50·1; H, 6·2; N, 6·45; $C_9H_{11}O_4N.H_2O$ requires: C, 50·2; H, 6·1; N, 6·5%.) Loss of weight on drying at 110° (1 mm.) 8·1%, the *monohydrate* requires 8·4%. The D-*amino-acid* had m.p. 265°, $[\alpha]_D^{18°}$ +7·9° in N-HCl (*c*, 3·0), $[\alpha]_D^{16°}$ +40·0°±3·0 in water (*c*, 0·15).

Hydantoin. The L-amino-acid was converted into the L-*hydantoin*; 0·5 g. of the amino-acid and KCNO (0·5 g.) were dissolved in hot water (30 ml.). The solution was heated on the water bath for 40 min., whilst H_2 was passed through the solution. 5N-HCl (7·5 ml.) was then added and heating continued for 2 hr. On cooling crystals appeared which were filtered off and, after treatment with charcoal, recrystallized from water. The L-*hydantoin* had m.p. 220–221°, $[\alpha]_D^{16°}$ −124·5° in 90% (v/v) ethanol (*c*, 0·92). (Found: N, 12·3. $C_{10}H_{10}O_4N_2$ requires N, 12·6%.)

METABOLIC EXPERIMENTS

Rats. Male and female rats of the Institute stock, weighing between 250 and 300 g., and fed ordinary rations, were used for these experiments. The amino-acids were mixed with the food, and allowance was made for material which had not been consumed. The analytical methods used have been described (Neuberger, 1947). The L-2:5-dihydroxyphenylalanine was given to 10 rats in doses ranging from 50 to 350 mg./100 g. body wt./day. Only two animals, receiving the highest doses, excreted a reducing substance which appeared to be unchanged amino-acid. It amounted to 8 and 10% respectively of the intake, as judged by the Briggs reaction. Identical results were obtained with urine samples which had been hydrolyzed with 0·5N- or 1·0N-HCl for 1 hr. at 100°. The D-amino-acid gave rise to the excretion of reducing substances in four animals which had received 250, 275, 300 and 325 mg./100 g. body wt./day. No reducing substance was excreted with doses of 100 and 150 mg./100 body wt./day. The amount of material excreted, as estimated by the Briggs reaction, amounted to 10, 12, 20 and 18% respectively of the intake. Hydrolysis with HCl, as described above, did not increase the amounts of reducing material.

Man. The L-amino-acid (5 g.) dissolved in warm water (500 ml.) was taken by a subject (A. N.) and urine collected for the next 48 hr. No reducing material could be detected in the urine, even after hydrolysis by acid. The same amount of the D-amino-acid produced reducing material in the urine collected during the first 8 hr. after ingestion. It amounted to about 0·8 g. A similar result was obtained on ingestion of 3 g. of the D-amino-acid; 0·6 g. of reducing material (in terms of the amino-acid) was excreted. No untoward symptoms were experienced.

Toxicity. The low solubility of the active amino-acid in water precluded the use of very large amounts of the compound in tests using a parenteral route. A cat (2·1 kg.) was given 30 ml. of a supersaturated 2·5% solution of the DL-amino-acid by stomach tube. No toxic symptoms were observed. Eight mice (20–30 g. wt.) were injected intraperitoneally with 0·15 ml. each of 0·5% solution of the L-amino-acid; no toxic symptoms were observed. Similar negative results were obtained on intravenous administration to rats and intraperitoneal administration to guinea pigs and rats.

SUMMARY

1. Gentisic aldehyde has been condensed with acetylglycine to give a mixture of an azlactone and a coumarin derivative. Both compounds have been converted to 2:5-dihydroxyphenylalanine and the corresponding α-keto acid.

2. The quinolic amino-acid has also been prepared from 2:5-dimethoxybenzaldehyde through the azlactone and the benzamidopropionic acid. The latter has been resolved with phenylethylamine and the two optical isomers of the free amino-acid been obtained.

3. It has been shown that both isomers are well metabolized by man and rat, the D-compound only slightly less efficiently than the L-isomeride. Both substances were shown to be non-toxic. Reasons are advanced for the assumption that L-2:5-dihydroxyphenylalanine is an intermediate in tyrosine metabolism.

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