[CONTRIBUTION FROM THE DEPARTMENT OF PHYSIOLOGY, THE UNIVERSITY OF ROCHESTER, SCHOOL OF MEDICINE AND DENTISTRY]

The Synthesis of the 2,4-Dihydroxymethylphenylalanines and the Possible Sites of the Linkages between Tyrosinase and Substrate[1]

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By the use of the 2,4-dihydroxymethylphenylalanines it has become possible to propose a theory for the sites of the linkages which exist between tyrosinase from the potato and a variety of compounds which serve as substrates, inhibitors, or are devoid of either activity. An excellent method for the synthesis of dimethoxytoluenes has been found in the methylation of phenyllithium compounds with dimethyl sulfate. Formylation of dimethoxybenzenes and dimethoxytoluenes by means of N-methyl-N-phenylformamide or N,N-dimethylformamide have been found to be excellent procedures in some cases and completely useless in others. *o*-Tyrosine has been produced by a two-step synthesis.

On an earlier occasion[2] we reported that in addition to tyrosine, the 2,3-, 2,5- and 3,4-dihydroxyphenylalanines could serve as substrate for tyrosinase obtained from the potato. Of the remaining three isomers, the 2,6- and the 3,5-dihydroxyphenylalanines were found to be neither substrates nor inhibitors, while 2,4-dihydroxyphenylalanine was a potent, reversible inhibitor of tyrosine for this enzyme.

Consideration of these findings led to speculation as to the sites of the linkages which might exist between the enzyme and its substrate. The inhibitory activity of 2,4-dihydroxyphenylalanine did not appear to be attributable to the *p*-hydroxy group because of its occurrence in tyrosine and in 3,4-dihydroxyphenylalanine, both of which are substrates, nor did it appear to be attributable to the *o*-hydroxy group since this group occurs in the substrates 2,3- and 2,5-dihydroxyphenylalanine and in the inert compound 2,6-dihydroxyphenylalanine. The latter contention has been supported by our findings that *o*-tyrosine has neither substrate[3] nor inhibitory properties. It must be concluded, therefore, that both the *o*- and *p*-hydroxy groups become linked to the enzyme through appropriate functional groups.

That the enzyme has two functional groups capable of linking to the *o*- and *p*-hydroxy groups appears to be confirmed by the structures of the substrate molecules, all of which have one or the other of these groups. It appears that the *o*-hydroxy–enzyme link is not sufficient to bring about oxidation unless the molecule is in an oxidation state which is readily convertible to an *o*- or *p*-quinone, since *o*-tyrosine and 2,6-dihydroxyphenylalanine are not substrates, while the 2,3- and 2,5-dihydroxyphenylalanines are. It also appears that the *p*-hydroxy–enzyme link is capable of initiating oxidation since *p*-tyrosine is oxidized. If linkage is made so that both the *o*-hydroxy–enzyme link and the *p*-hydroxy–enzyme link are accomplished, the enzyme is reversibly inhibited.

One might conclude, therefore, that tyrosinase has at least three functional groups which have structural proximity. One of these links to the α-amino group. The second can link to an *o*-hydroxy group but can accomplish oxidation only under special conditions of substrate structure. The third can link to the *p*-hydroxy group and is capable of initiating oxidation. In view of the fact that this theory imposes certain structural limitations on the substrate molecule, it was considered to be one which could be tested by studying the action of the enzyme on various compounds containing these three groups, and one non-functional group to act as a steric barrier.

We have synthesized 3-methyl-, 5-methyl- and 6-methyl-2,4-dihydroxyphenylanine and have tested them for substrate and inhibitory properties and have found their activities to support the concepts outlined above. 2,4-Dihydroxy-3-methylphenylalanine is a substrate. Its rate of oxidation is about the same as that of tyrosine but the color produced is brick-red. 2,4-Dihydroxy-5-methylphenylalanine is completely inert. 2,4-Dihydroxy-6-methylphenylalanine is a somewhat less potent reversible inhibitor than the basic 2,4-dihydroxyphenylalanine.

The evidence suggests that the methyl group in the 3-methylamino acid sterically prevents simultaneous linkage to both the *o*- and *p*-hydroxy groups. Since the compound cannot be converted directly to a quinone, a linkage of the *o*-hydroxy–enzyme type, if it is formed at all, accomplishes nothing. The *p*-hydroxy–enzyme link is like that of *p*-tyrosine and the compound is readily oxidized, presumably in the 5-position. The methyl group in the 5-methylamino acid also sterically prevents simultaneous linkage to both the *o*-hydroxy and *p*-hydroxy groups but, furthermore, it prevents the *p*-hydroxy–enzyme link altogether. The *o*-hydroxy–enzyme link prevents free rotation of the molecule but is not capable of initiating oxidation. The compound should, and does, behave like *o*-tyrosine and 2,6-dihydroxyphenylalanine. The methyl group in the 6-methylamino acid is sufficiently removed from the linkage sites to permit the linkage to both the *o*- and *p*-hydroxy groups. That it is not devoid of steric interference with the linkages is evidenced by its reduced potency as an inhibitor.

2,4-Dimethoxy- and 2,6-dimethoxytoluene were both prepared in large quantity by means of the direct methylation of the appropriate phenyllithium compounds with dimethyl sulfate.

The dimethoxymethylbenzaldehydes were prepared from the toluenes by the use of phosphoryl

(1) This work was supported in part by a Grant-in-Aid from the American Medical Association, Council on Pharmacy and Chemistry, Grant Number 123.

(2) J. P. Lambooy, THIS JOURNAL, **76**, 133 (1954).

(3) A. B. Gutmann, *Fermentforschung*, **9**, 117 (1928), had reported that *o*- and *m*-tyrosine were devoid of substrate activity. The absence of experimental data made it impossible to know whether *o*-tyrosine had inhibitory properties.

chloride and either N-methyl-N-phenylformamide or N,N-dimethylformamide. These procedures produced 2,4-dimethoxy-3-methylbenzaldehyde and 2,4-dimethoxy-5-methylbenzaldehyde in excellent yields, conveniently and safely, but were inferior to the Gattermann–Adams cyanide synthesis for the preparation of 2,4-dimethoxy-6-methylbenzaldehyde. The procedures employing the formamides were applied to several dimethoxybenzenes and toluenes with yields varying from 0 to 94%, depending on the positions of the substituents.

Brief descriptions for variations in the cyanide syntheses have been included because of their contribution to better yields or purity of products.

The dimethoxymethylbenzaldehydes were converted to the azlactones by means of the Erlenmeyer reaction. The azlactones were converted to the acrylic acids and the latter reduced to the propionic acids by means of Raney nickel-catalyzed hydrogenation. The hydrogenation was somewhat unusual in that the N benzamidoacrylic acids were reduced to the N-cyclohexanecarboxamidopropionic acids. The propionic acids were converted to the amino acids by means of hydrochloric acid in a sealed tube at 150°.

o-Tyrosine has been prepared in good yield and excellent quality by a convenient two-step synthesis.

Experimental

By means of an extension of the methylation procedure described by Cullinane and Philpott,[4] one-half mole lots of the following compounds were methylated to their respective products in the yields indicated: 2,4-dihydroxybenzaldehyde, 97%, m.p. 71–72°[5]; 4-bromoresorcinol,[6] 97%, b.p. 147° (18 mm.)[7]; 3,5-dihydroxytoluene, 96%, b.p. 104–105° (12 mm.), and 2,4-dihydroxy-6-methylbenzaldehyde, 93%, m.p. 67–68°.[8]

General Procedure a.—To 27 g. (0.2 mole) of N-methyl-N-phenylformamide was added dropwise 30.6 g. (0.2 mole) of phosphoryl chloride, followed by the dropwise addition of 13.8 g. (0.1 mole) of *m*-dimethoxybenzene. After the original exothermic reaction had subsided, the mixture was heated on the steam-bath for 2 hours. The product was poured with stirring into 300 g. of ice, and, after crystallization had begun, diluted to one liter and placed in the cold for 12 hours. The product was recrystallized from ethanol to yield 11.9 g. (72%) of colorless crystals, m.p. 71–72°.

General Procedure b.—To 29.2 g. (31 ml., 0.4 mole) of N,N-dimethylformamide cooled in an ice-bath was added dropwise 30.6 g. (18.3 ml., 0.2 mole) of phosphoryl chloride. The *m*-dimethoxybenzene, 13.8 g. (0.1 mole), was then added and the ice-bath replaced by a steam-bath. After the 2-hour heating period the reaction mixture was treated as in (a) above, to yield 11.2 g. (68%) of material, m.p. 70–72°.

The following dimethoxy compounds were converted to the designated benzaldehydes in the yields indicated for general procedures (a) and (b), respectively: *p*-dimethoxybenzene to 2,5-dimethoxy-, 7, 0%; *o*-dimethoxybenzene to 3,4-dimethoxy-, 52, 11%; 2,6-dimethoxytoluene to 2,4-dimethoxy-3-methyl-, 81, 89%; 2,4-dimethoxytoluene to 2,4-dimethoxy-5-methyl-, 90, 93%; 3,5-dimethoxytoluene to 2,4-dimethoxy-6-methyl-, 70, 51%.

2,4-Dimethoxytoluene.—2,4-Dimethoxybenzaldehyde was converted to the hydrazone in a yield of 97%, m.p. 202–203°. The hydrazone was reduced by the procedure described by Cram[8] except that the decomposition was carried out at 145° instead of 185°, a change found to be necessary to avoid extensive demethylation.

2,4-Dimethoxybromobenzene in 1.0 mole lots was converted to the lithium compound by means of phenyllithium and methylated to 2,4-dimethoxytoluene in yields of 92%, b.p. 101–102° (12 mm.), by essentially the same procedure as that used for the preparation of 2,6-dimethoxytoluene. The yield was reduced when lithium was used to metalate the 2,4-dimethoxybromobenzene. The Clemmensen reduction of 2,4-dimethoxybenzaldehyde produced yields of only 62% of the toluene.

2,6-Dimethoxytoluene.—Phenyllithium was prepared in the usual manner from 18.6 g. (2.68 moles) of lithium, 750 ml. of absolute ether and 210 g. (1.34 moles) of bromobenzene. To this solution was added 138 g. (1.0 mole) of *m*-dimethoxybenzene and the transfer and storage were identical with those described for the preparation of 2,6-dimethoxybenzaldehyde.[2] After the 3-day storage, 169 g. (125 ml., 1.34 moles) of dimethyl sulfate was added, with swirling, over a period of 2 hours. The contents were refluxed for 1 hour and poured into ice and water and the product isolated by ether extraction. The product was distilled from a Claissen flask, only that portion boiling above 110° (20 mm.) being collected to yield 140–143 g. The solid distillate was melted, 50 ml. of *n*-pentane was added and the solution placed in the cold. After 2–3 days of cold storage the liquid portion was decanted from the very large crystals and the product washed with 20 ml. of cold *n*-pentane. The crystals were dried in the cold to yield 88–92 g. of 2,6-dimethoxytoluene, m.p. 39–40°.[9] The decanted liquid portion and wash solution were freed of *n*-pentane and fractionated, only that portion which distilled 110–113° (20 mm.) was collected. This distillate was treated with 10 ml. of *n*-pentane and processed as before, being eventually washed with 5 ml. of *n*-pentane, to yield 24 g. of the toluene for a total yield of 112–116 g., m.p. 39° (74–76%).[10] When several lots of the toluene were combined and fractionated, the product had b.p. 97–99° (15 mm.) and m.p. 39–40°.

The Clemmensen reduction of 2,6-dimethoxybenzaldehyde[2] yielded only 44% of the desired toluene.

2,4-Dimethoxy-3-methylbenzaldehyde.—This material is most conveniently prepared by general procedure b. The following procedure is included because of convenient variations in the cyanide synthesis. 2,6-Dimethoxytoluene, 25 g. (0.164 mole), was converted to the desired aldehyde by the procedure described by Adams and Montgomery[11] for the preparation of 2,4-dimethoxybenzaldehyde with the following variations. Zinc cyanide,[12] 40 g. (0.34 mole), in 200 ml. of dry benzene was decomposed by the introduction of hydrogen chloride for 1 hour, then 38 g. (0.39 mole) of anhydrous aluminum chloride was added and the gas introduction continued for 0.5 hour. The toluene dissolved in 25 ml. of benzene was added in 5-ml. portions over a period of 20 min. and the mixture was warmed to 45–50° while the gas addition was continued for 4 hours. The reaction mixture was poured into 800 cc. of ice, filtered and washed with 100 ml. of water. The precipitate was refluxed for 10 min. with 500 ml. of 10% hydrochloric acid, and extracted with ether. The product was dissolved in 160 ml. of *n*-hexane, decolorized, and placed in the cold, to yield 20.5 g. (72%) of material as long white needles, m.p. 53–54°. Recrystallization raised the melting point to 54–55°. The material was characterized by its conversion to 2,4-dihydroxy-3-methylbenzaldehyde.

Anal. Calcd. for $C_{10}H_{12}O_3$: C, 66.7; H, 6.7. Found: C, 66.7; H, 6.9.

2,4-Dimethoxy-5-methylbenzaldehyde.—This material is most conveniently prepared by general procedure b. The following procedure is included because of convenient varia-

(4) N. M. Cullinane and D. Philpott, *J. Chem. Soc.*, 1763 (1929).

(5) All melting and decomposition points were determined on calibrated thermometers.

(6) R. B. Sandin and R. A. McKee, *Org. Syntheses*, **17**, 23 (1937).

(7) G. P. Rice, THIS JOURNAL, **48**, 3128 (1936), obtained a product in 28% yield of b.p. 135° (18 mm.).

(8) D. J. Cram, *ibid.*, **70**, 4243 (1948), reported m.p. 64–65°

(9) S. Sibata, *C. A.*, **33**, 8183 (1939), reported, m.p. 39°.

(10) The actual yield is undoubtedly higher but the separation of the toluene (b.p. 111–113° (20 mm.)) from the unreacted *m*-dimethoxybenzene (b.p. 104–105° (20 mm.)) is difficult. The described procedure is convenient.

(11) R. Adams and E. Montgomery, THIS JOURNAL, **46**, 1518 (1924). If the reaction is run as described in this report, the mixture sets into a solid mass which is broken up with very great difficulty and a much reduced yield is obtained.

(12) Technical grade zinc cyanide powder, City Chemical Corporation, New York, was found to be suitable for use as received.

tions in the cyanide synthesis. 2,4-Dimethoxytoluene, 25 g. (0.164 mole), was converted to the desired aldehyde by the procedure described[11] for the preparation of 2,4-dimethoxybenzaldehyde, with the following variations. The reaction product was poured into a mixture of 143 ml. of hydrochloric acid and 400 g. of ice and filtered. The precipitate was washed with 100 ml. of 10% hydrochloric acid and then with 100 ml. of ether. The product was decomposed by refluxing for 10 min. with 500 ml. of 10% hydrochloric acid and placed in the cold. The product was recrystallized from 200 ml. of ethanol to yield 21.9 g. (74%) of material, m.p. 120–121°.[13]

2,4-Dimethoxy-6-methylbenzaldehyde. 2,4-Dihydroxy-6-methylbenzaldehyde.—3,5-Dihydroxytoluene, 20 g. (0.16 mole), zinc cyanide, 33 g. (0.28 mole) and 200 ml. of absolute ether were treated as described[14] for the preparation of orcinol aldehyde with the following variations. At the end of the reaction the original ether solution was decanted and the paste-like reaction mixture was washed 5 times by stirring with 100-ml. portions of stock ether. The paste was dissolved in 350 ml. of water and boiled until it became a semi-solid mass, and then placed in the cold. Filtration produced 23.5 to 23.7 g. (96–97%)[15] of material, m.p. 181–182°.

Preparation of the 4-(2,4-Dimethoxymethylbenzylidine)-2-phenyl-5-oxazolones.[16]—A mixture of the appropriate aldehyde, 9.0 g. (0.05 mole), 13.3 g. (0.075 mole) of benzoylglycine, 13.5 g. (0.159 mole) of freshly fused sodium acetate, 40 ml. of acetic anhydride and 40 ml. of glacial acetic acid, was heated in a boiling water-bath for 0.5 hour. The solvents were removed under reduced pressure until the mixture solidified. The contents were cooled and triturated in 300 ml. of water. Several hours later the product was filtered and recrystallized from the minimum amount of ethanol,[17] to yield the bulk of the products. The alcoholic filtrates were evaporated to 50 ml., 15 ml. of 0.5 *N* sodium hydroxide was added and the mixture heated on the steam-bath for 0.5 hour. The alkaline solution was decolorized and 100–125 ml. of water added and the material cooled. The unreacted aldehydes were thus recovered in slightly colored form and were suitable for reuse in the procedure. The yields of azlactones are reported before and after correction for the recovered aldehyde.

2,4-Dimethoxy-3-methylbenzaldehyde yielded 7.2 g. (45%) (59%), of azlactone as yellow needles, m.p. 170–171° (analytical sample, m.p. 173–175°).

Anal. Calcd. for $C_{19}H_{17}NO_4$: C, 70.6; H, 5.3; N, 4.3. Found: C, 70.9; H, 5.7; N, 4.6.

2,4-Dimethoxy-5-methylbenzaldehyde yielded 9.2 g. (58%), (80%), of azlactone as yellow needles or orange prisms, m.p. 205–208° (analytical sample, m.p. 207–208°).

Anal. Calcd. for $C_{19}H_{17}NO_4$: C, 70.6; H, 5.3; N, 4.3. Found: C, 70.8; H, 5.3; N, 4.4.

The 2,4-dimethoxy-6-methylbenzaldehyde yielded 4.0 g. (25%), (74%), as yellow needles, m.p. 165° (analytical sample, m.p. 166–167°).

Anal. Calcd. for $C_{19}H_{17}NO_4$: C, 70.6; H, 5.3; N, 4.3. Found: C, 70.7; H, 5.3; N, 4.4.

Salicylaldehyde, 30.5 g. (0.25 mole), 46.0 g. (0.26 mole) benzoyl glycine, 28 g. of sodium acetate and 100 ml. of acetic anhydride were treated essentially as described for the preparation of 2-phenyl-4-(2,5-diacetoxybenzal)-5-oxazolone (a),[18] to produce 46.0 g. of a crude mixture of 4-(2-acetoxybenzylidine)-2-phenyl-5-oxazolone and 2-keto-3-benzamidocoumarin. This material was converted directly to *o*-tyrosine.

Preparation of the 2-Benzamido-3-(2,4-dimethoxymethylphenyl)-acrylic Acids.—The azlactones were suspended in 500 ml. of ethanol and heated to the boiling point on the steam-bath. Warm (70–80°) 0.5 *N* sodium hydroxide, 200 ml., was added and the mixture heated for 0.5 hour. Water, 300 ml. was added and the solution boiled to remove the ethanol. The solution was kept in an ice-water bath 0.5 hour before[19] and after acidification with 15 ml. of 36% hydrochloric acid. The product was filtered, washed with water, and recrystallized from ethanol.

4-(2,4-Dimethoxy-3-methylbenzylidine)-2-phenyl-5-oxazolone, 21.0 g. (0.065 mole), produced 21.0 g. (95%) as white needles, m.p. 225–226° dec. (analytical sample, m.p. 225–226° dec.).

Anal. Calcd. for $C_{19}H_{19}NO_5$: C, 66.9; H, 5.6; N, 4.1. Found: C, 67.1; H, 5.8; N, 3.9.

4-(2,4-Dimethoxy-5-methylbenzylidine)-2-phenyl-5-oxazolone, 27.9 g. (0.086 mole), produced 28.3 g. (96%) as white needles, m.p. 245° dec. (analytical sample, m.p. 245–246° dec.).

Anal. Calcd. for $C_{19}H_{19}NO_5$: C, 66.9; H, 5.6; N, 4.1. Found: C, 67.2; H, 5.8; N, 4.1.

4-(2,4-Dimethoxy-6-methylbenzylidine)-2-phenyl-5-oxazolone, 27.5 g. (0.085 mole), produced 27.0 g. (93%) of white needles, m.p. 213–215° dec. (analytical sample, m.p. 215–217° dec.).

Anal. Calcd. for $C_{19}H_{19}NO_5$: C, 66.9; H, 5.6; N, 4.1. Found: C, 66.9; H, 5.8; N, 3.9.

Preparation of the 2-Cyclohexanecarboxamido-3-(2,4-dimethoxymethylphenyl)-propionic Acids.—The acrylic acid, 130 ml. of 0.5 *N* sodium hydroxide and 40 g. of well settled Raney nickel were hydrogenated at 80–85 atmospheres of hydrogen, at 60°[19] for 48 hours.[20] Following filtration the solution was chilled[19] to 0° for 0.5 hour before and after acidification with 6 *N* hydrochloric acid. The propionic acids were recrystallized from 50% (v./v.) acetic acid.

2-Benzamido-3-(2,4-dimethoxy-3-methylphenyl)-acrylic acid, 13.6 g. (0.04 mole), yielded a gummy product on acidification. Following a single recrystallization 13.1 g. (94%) was obtained as white crystals, m.p. 138–139°. The analytical sample was recrystallized from a mixture of benzene and *n*-hexane to produce colorless crystals, m.p. 139–141°.

Anal. Calcd. for $C_{19}H_{27}NO_5$: C, 65.3; H, 7.8; N, 4.0. Found: C, 65.3; H, 7.5; N, 4.2.

2-Benzamido-3-(2,4-dimethoxy-5-methylphenyl)-acrylic acid, 17.1 g. (0.05 mole), cooled after acidification,[19] yielded 17.2 g. (98%), as pale yellow needles, m.p. 186–187° (the colorless analytical sample, m.p. 186–187°).

Anal. Calcd. for $C_{19}H_{27}NO_5$: C, 65.3; H, 7.8; N, 4.0. Found: C, 65.8; H, 7.7; N, 4.1.

2-Benzamide-3-(2,4-dimethoxy-6-methylphenyl)-acrylic acid, 13.6 g. (0.04 mole), yielded 14.4 g. (102%) as colorless crystals which contain a molecule of water of crystallization and which soften at about 120–123° and melt with effervescence at 129–130°. The water is lost over phosphorus pentoxide at reduced pressures. The analytical sample was dried for successive 24-hour periods at 57, 78 and 100° and 3 mm. to yield colorless crystalline material, m.p. 157–158°.

Anal. Calcd. for $C_{19}H_{27}NO_5$: C, 65.3; H, 7.8; N, 4.0; H_2O, 5.0. Found: C, 65.7; H, 7.7; N, 4.0; H_2O, 5.5.

Preparation of the 2,4-Dihydroxymethylphenylalanines.—Three grams of the propionic acid and 25 ml. of concentrated hydrochloric acid were heated in a sealed Carius tube for 2 hours at 150°. The contents of two or three tubes were diluted with two volumes of water and extracted with ether.[21] The water phase was evaporated to dryness under reduced pressure, hydrogen being drawn in through the capillary. The residue was dissolved in 50 ml. of water and adjusted to *p*H 7 to 8 with concentrated ammonium hydroxide and evaporated to dryness as above. The residue was suspended in 15 to 20 ml. of water and filtered to yield the

(13) D. J. Cram, THIS JOURNAL, **72**, 595 (1950), reports, m.p. 117–118°.

(14) R. Adams and I. Levine, *ibid.*, **45**, 2373 (1923).

(15) Ref. 8 reported a yield of 71%, m.p. 181–182°.

(16) Several variations of this synthesis were used. The one given was best from the viewpoint of over-all yield and quality of the recovered aldehyde. The direct yield of azlactone was somewhat greater in some other cases but the unused aldehyde was recovered in only small amounts.

(17) The 5-methylazlactone is always more conveniently recrystallized from *n*-butanol; with larger batches of the 3-methylazlactone, *n*-butanol is also more convenient.

(18) J. P. Lambooy, THIS JOURNAL, **71**, 3758 (1949).

(19) The sodium salts of the 5-methylacrylic acid and propionic acid are relatively insoluble below 40–50°. For uniformity, all reductions were run at 60°. For this reason filtration to remove the catalyst and the subsequent acidification of the 5-methylpropionic acid was done at about 60°.

(20) With the conditions and lot of catalyst used this reduction time appeared to be necessary for the 5-methylacrylic acid. For uniformity, the time was made the same in all cases.

(21) Cyclohexanecarboxylic acid, m.p. 30–31°, and b.p. 120–122° (14–15 mm.), was isolated from these ether extracts.

amino acid in crude form. The product required two or three recrystallizations from water containing a trace of sulfur dioxide before it was pure.

2-Cyclohexanecarboxamido-3-(2,4-dimethoxy-3-methylphenyl)-propionic acid, 9.0 g. (0.026 mole), yielded 1.09 g. (20%) as microscopic colorless needles, m.p. 248° dec. (analytical sample, m.p. 254° dec.).

Anal. Calcd. for $C_{16}H_{13}NO_4$: C, 56.9; H, 6.2; N, 6.6. Found: C, 56.9; H, 6.4; N, 6.5.

2-Cyclohexanecarboxamido-3-(2,4-dimethoxy-5-methylphenyl)-propionic acid, 6.0 g. (0.017 mole), yielded 2.31 g. (64%) as colorless prisms, m.p. 256° dec. (analytical sample, m.p. 257° dec.).

Anal. Calcd. for $C_{16}H_{13}NO_4$: C, 56.9; H, 6.2; N, 6.6. Found: C, 57.2; H, 6.0; N, 6.9.

2-Cyclohexanecarboxamido-3-(2,4-dimethoxy-6-methylphenyl)-propionic acid, 9.0 g. (0.026 mole), yielded 1.60 g. (30%) as colorless prisms, m.p. 255–256° dec. (analytical sample, m.p. 260° dec.).

Anal. Calcd. for $C_{16}H_{13}NO_4$: C, 56.9; H, 6.2; N, 6.6. Found: C, 57.2; H, 6.3; N, 7.0.

The 46.0 g. of the crude mixture of 2-keto-3-benzamidocoumarin and 4-(2-acetoxybenzylidine)-2-phenyl-5-oxazolone, 6.0 g. of red phosphorus and 175 ml. each of hydriodic acid (sp. gr. 1.7) and glacial acetic acid were refluxed for 2 hours under an atmosphere of hydrogen. The mixture was filtered and evaporated under reduced pressure, hydrogen being drawn in through the capillary. The residue was suspended in 250 ml. of water, filtered and extracted with ether. The water phase was evaporated as above and the residual sirup dissolved in 150 ml. of water, the *p*H adjusted to approximately 7 by means of concentrated ammonium hydroxide solution and the suspension stored in the refrigerator. The product was collected, dissolved in 900 ml. of boiling water, decolorized and evaporated as above to about 500 ml. and refrigerated. The collected precipitate was washed on the filter with 25 ml. of water containing SO_2 and dried to produce 16.1 g. (36%) of *o*-tyrosine, m.p. 246° dec. as colorless crystalline material. The product was dissolved in 800 ml. of water and boiled on the hot plate until concentrated to 400 ml., treated with decolorizing carbon and refrigerated. The precipitate consisted of large crystals, m.p. 265° dec.[22] weighing 12.9 g. or 29% of the theoretical amount based on the quantity of salicylaldehyde used.

Paper Chromatography.—The R_f values of the amino acids were determined for the phenol–water system. Under the conditions which prevailed during the determination by the descending technique, L(−)tyrosine had an R_f value of 0.58; 2,4-dihydroxyphenylalanine, the 3-methyl-, 5-methyl-, 6-methyl-, 2,4-dihydroxyphenylalanines and *o*-tyrosine had R_f values of 0.37,[2] 0.50, 0.50, 0.44 and 0.71, respectively.

Enzyme Activity Determinations.—The enzyme studies were done essentially like those reported on a previous occasion.[2] 2,4-Dihydroxy-3-methylphenylalanine was used at levels of 0.1 to 2.0 mg. and when compared to tubes containing one-half these levels of L(−)tyrosine it was found that the 3-methylamino acid was oxidized at a somewhat greater rate than tyrosine, but to a brick-red color. The same levels of 2,4-dihydroxy-5-methylphenylalanine were used and all tubes gave the same reading after 6 hours as were given by the enzyme blanks. When tyrosine was also added the color production was equivalent to the tyrosine added. Graded levels of 2,4-dihydroxy-6-methylphenylalanine (0.1 to 1.5 mg.) progressively inhibited the oxidation of 0.25 mg. of L(−)tyrosine. The inhibition caused by 0.5 mg. of 6-methylamino acid was progressively relieved by the addition of graded amounts (0.15 to 1.0 mg.) of tyrosine.

(22) Previous reports of the m.p. were between 249 and 251° dec.

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Investigation of the Chemical Nature of Gonyleptidine

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The major component of the antibiotic gonyleptidine, a yellow pigment from the secretion of a South American arachnid, was identified by isolation of the hydroquinone diacetate as 2,3-dimethyl-1,4-quinone. Because of correspondence of infrared absorption bands with those of gonyleptidine, and because of even higher bacteriostatic potency, 2,5-dimethyl-1,4-quinone and 2,3,5-trimethyl-1,4-quinone seemed likely minor companions, and various methods of fractionation were tested on known mixtures of the three synthetic quinones. That finally applied to 115 mg. of gonyleptidine involved, in the first step, reaction with 2,3-dimethylbutadiene for selective conversion of 2,3-dimethyl-1,4-quinone to an adduct, which remained in the neutral fraction when the unreacted quinones were reduced with hydrosulfite and extracted with alkali. After reoxidation, the quinone mixture was submitted to Thiele acetoxylation. The 2,5-dimethyl-1,4-quinone present yielded the non-steam-volatile 2,5-dimethyl-1,3,4-triacetoxybenzene, and 2,3,5-trimethyl-1,4-quinone was isolated from the steam distillate. Thioacetic and β-thiopropionic acid derivatives of the alkyl-1,4-quinone were prepared incidentally and some of them have been found to be bacteriostatic. New observations concerning the scope of the Thiele reaction are presented.

Clemente Estable and associates of the Instituto de Investigación de Ciencias Biológicas, Ministerio de Salud Pública, Montevideo, Uruguay, discovered that a yellow aqueous fluid secreted by the South American arachnid *Gonyleptide* has remarkable antibiotic properties.[1] The yellow pigment, named gonyleptidine, is bacteriostatic, *in vitro*, against Gram positive and Gram negative bacteria and protozoa. The initial discovery was made in consequence of striking biological actions exerted by minute amounts of material reaching microörganisms at one site in the laboratory by air transport from arachnids at a distant site.

Withdrawal of secretion from an arachnid by capillary pipet affords only about 0.01 ml. of a yellow aqueous suspension containing 2–3 mg. of pigment per ml., and after one or two repetitions of the process the arachnid fails to yield significant further amounts of fluid. Thus the amount of antibiotic pigment that can be collected in a given season from thousands of arachnids is small. The research thus reached a point where identification of the active principle or principles of gonyleptidine become imperative. Exploratory investigations conducted by one of us at Montevideo established that on distillation of extract from the frozen state gonyleptidine distils first as a bright yellow crystalline substance of melting point about 12° and is followed by a water solution of the pigment. The substance shows selective ultraviolet absorption at 255 mμ with the extinction coefficient $E^{1\%}$ 1400 (water). Antibiotic activity against various micro-

(1) C. Estable, M. I. Ardao, N. P. Brasil and L. F. Fieser, THIS JOURNAL, **77**, 4942 (1955).