Table 2. *Bile pigments from crystalline human oxyhaemoglobin*

Specimen	Weight of crystalline oxyhaemoglobin (g.)	Total yield of bile pigments (μ g.)
1	3.7	32.1
2	3.7	24.0
3	3.8	32.6
4	4.3	45.9

DISCUSSION

It is evident from this investigation that the bile pigment precursor content of human erythrocytes is not greater than that associated with the corresponding amount of crystalline oxyhaemoglobin. Such a result could arise if either of two quite different conditions obtain. The minute yields of bile pigment might be caused artificially by the drastic chemical processes involved. Alternatively, bile pigment precursor may pass along with oxyhaemoglobin through all the steps of purification into the crystalline product, maintaining the original quantitative relations with the mother pigment which obtained in the original erythrocytes. This latter hypothesis appears unlikely, but no means are available at present of deciding which is the true interpretation. The suggestion that early changes in the breakdown of haemoglobin to bile pigments are initiated during circulation receives little support

from the present experiments. It is possible that a more fruitful search for intermediates of pigment metabolism could be made in situations where the reticulo-endothelial system is overloaded with haemoglobin catabolic products, as in the spleen of patients suffering from haemolytic jaundice or polycythaemia.

SUMMARY

1. An anaerobic method for the study of bile pigment precursor in human erythrocytes and in crystalline human haemoglobin is described.

2. The bile pigment precursor content of human erythrocytes was found to be 0.17 % of the contained haemoglobin. Bile pigment precursor also constituted 0.21 % of crystalline human oxyhaemoglobin.

3. A fluorimetric method for measurement of bile pigments has been developed. Its special advantages are: (a) it has a high sensitivity, adequate for the small quantities of pigments encountered in this problem; (b) it is specific, with minimal interference from other pigments; (c) it is not dependent on the preparation of a pure bilipurpurin standard.

4. The experiments provide little support for the hypothesis that early degradative changes of oxyhaemoglobin are initiated in the circulating erythrocyte.

This work was carried out during the tenure by one of us (C. G.) of the Sybil Mary Pilkington Fellowship.

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The Action of Enzymes on Chlorophenylalanines

By H. BLASCHKO AND JEAN STIVEN

The Department of Pharmacology, University of Oxford

(Received 4 August 1949)

The enzymic oxidation and decarboxylation of tyrosine and its homologues has recently been studied (Blaschko, Holton & Sloane-Stanley, 1949; Blaschko, 1949; Sloane-Stanley, 1949). It was found that *o*-hydroxyphenylalanine and *m*-hydroxyphenylalanine were decarboxylated by extracts of mammalian tissues, which contain the enzyme 3:4-dihydroxyphenylalanine (DOPA) decarboxylase, an enzyme known not to act on tyrosine. On the

other hand, acetone-dried preparations of *Streptococcus faecalis* R, containing the enzyme tyrosine decarboxylase, acted not only on tyrosine, but also on *m*-hydroxyphenylalanine; they did not decarboxylate *o*-hydroxyphenylalanine. The amino-acid oxidases showed a lesser degree of specificity: the L-amino-acid oxidase of cobra venom and the D-amino-acid oxidase of pig kidney oxidized all three hydroxyphenylalanines.

The position of the phenolic hydroxyl group and its importance for the substrate specificity of the amino-acid decarboxylases has recently been discussed elsewhere (Blaschko, 1950). It has been suggested that the reaction between the enzyme protein and the substrate involves a reaction through the phenolic hydroxyl group. This would explain why each decarboxylase accepts as substrates two isomers in which the phenolic hydroxyl group occupies one of two vicinal positions on the benzene ring and is unable to react with the third isomer.

It was, therefore, of interest to find out if in the decarboxylation the phenolic hydroxyl groups are essential for the occurrence of the enzyme-substrate reaction or if they can be replaced by another polar group of similar dimensions. It was for this reason that the monochlorophenylalanines have been examined as possible substrates of amino-acid decarboxylases. At the same time, the enzymic oxidation of these compounds by amino-acid oxidases has been studied.

MATERIALS AND METHODS

The preparation of *o*-, *m*- and *p*-chlorophenylalanine hydrochlorides. The chlorophenylalanine hydrochlorides were prepared by condensing the appropriate chlorobenzaldehyde with hippuric acid and reducing the oxazalone with hydriodic acid (Lamb & Robson, 1931); the resulting *N*-benzoylchlorophenylalanine was then hydrolysed with dilute HCl (Friedmann & Maase, 1910). In Lamb & Robson's method HI is used for both the reduction and the hydrolysis, but this method gave tarry products which were difficult to purify. The preparation of *o*-chlorophenylalanine hydrochloride is described in detail as it has not been prepared before. The *m*-chloro (Flatow, 1910) and *p*-chloro (Friedmann & Maase, 1910) compounds were prepared in an exactly similar way.

o-Chlorobenzaldehyde (14 g.) was condensed with hippuric acid (18 g.) by means of acetic anhydride (20 g., 22 ml.) and sodium acetate (8 g.) at 100°. *o*-Chlorobenzoxazalone crystallized from glacial acetic acid and melted at 158°. (Found: C, 67.2; H, 3.4. $C_{16}H_{10}O_2NCl$ requires C, 67.7; H, 3.5%). A mixture of the oxazalone (11.4 g.), glacial acetic acid (70 g.), HI (1.6 ml., sp.gr. 1.7) and red phosphorus (3 g.) was boiled for 1 hr. and then filtered while hot; the phosphorus was washed with hot acetic acid and the total filtrate evaporated *in vacuo*. Water was added and the evaporation repeated. The solid residue was dissolved in the minimum of hot ethanol and the solution boiled with charcoal and filtered; the filtrate was diluted with hot water until it became cloudy and then left overnight when *N*-benzoyl-*o*-chlorophenylalanine separated; it was recrystallized from glacial acetic acid, m.p. 206°. (Found: C, 63.7; H, 4.4. $C_{16}H_{14}O_2NCl$ requires C, 63.3; H, 4.6%). The benzoyl group was removed by hydrolysis with dilute HCl as described by Friedmann & Maase (1910). *o*-Chlorophenylalanine hydrochloride crystallized from water containing HCl, m.p. 240°. (Found: C, 45.2; H, 4.8. $C_9H_{11}O_2NCl_2$ requires C, 45.7; H, 4.7%).

Solutions of the three DL-chlorophenylalanine hydrochlorides were neutralized before use; the free amino-acids

are water soluble, but, as in the corresponding series of the hydroxyphenylalanines, the para-chloro derivative is the least soluble. In some of the experiments with *p*-chlorophenylalanine, it was noted that the amino-acid, which had been dissolved by heating, crystallized out on cooling in the manometer flasks.

Extracts of guinea pig kidney were prepared, as previously described (Blaschko, 1949). These extracts contain L-DOPA decarboxylase. The preparation of *Streptococcus faecalis* was an acetone-dried powder grown on a medium deficient in vitamin B₆; in the presence of pyridoxal and adenosinetriphosphoric acid (ATP) this preparation has very high L-tyrosine decarboxylase activity. The preparation was one made by Dr G. H. Sloane-Stanley, of this Department, about 9 months earlier. Stored in a desiccator at 0°, it had retained its activity; it was used as described by Sloane-Stanley (1949).

The cobra venom used for this work is a specimen from the collection of the late Sir Thomas Fraser in Edinburgh. It was given to the author by Prof. J. H. Gaddum and is labelled: 'Cobra venom, Capt Elliot, Madras, 1899'. It is worth noting that the L-amino-acid oxidase activity of this 50-year-old specimen is of the same order as that of recently collected venom which was used in a previous study (Blaschko, 1949) and which the author obtained from Dr C. H. Kellaway. The extract from an acetone-dried powder of pig kidney for the study of D-amino-acid oxidase was prepared as already described (Blaschko, 1949).

EXPERIMENTAL AND RESULTS

Decarboxylases

Guinea pig kidney. The extracts prepared had a high activity with L-3:4-dihydroxyphenylalanine as substrate. In each experiment four conical manometer flasks were used; the main compartment of each flask held 1.0 ml. guinea-pig kidney extract, containing the equivalent of 500 mg. of fresh tissue, plus 0.6 ml. of 0.067 M-sodium phosphate buffer of pH 7.4. The side bulbs contained:

Flask no. ...	...	1	2	3	4
Water (ml.)		0.4	0.2	0.2	—
0.02 M-L-3:4-dihydroxyphenylalanine (ml.)		—	0.2	—	0.2
0.04 M-DL-chlorophenylalanine (ml.)		—	—	0.2	0.2

In each experiment the gas phase was N₂. The formation of CO₂ in flasks 2 and 4 was rapid in each experiment and the reaction had practically gone to completion within the first 9 min. There was no formation of CO₂ with any of the three chlorophenylalanines, nor was there any indication that the presence of the chloro derivatives affected the rate of decarboxylation of L-3:4-dihydroxyphenylalanine.

Streptococcus faecalis R. Two experiments were carried out with an acetone-dried preparation of bacteria grown on the vitamin B₆-deficient medium described by Sloane-Stanley (1949). Each manometer flask contained 0.4 ml. of an aqueous suspension of the bacteria, containing 2 mg. of the dried powder, 0.1 ml. of a solution containing 1 mg. of ATP, 1.0 ml. of a pyridoxal hydrochloride solution (containing 10 µg. of pyridoxal), 0.2 ml. of a M-sodium acetate

buffer of pH 5.5, plus 0.9 ml. water. In the first experiment the side bulb of the first flask contained 0.4 ml. water, the second 0.4 ml. of a 0.04M-suspension of L-tyrosine; the other three flasks contained 0.4 ml. each of a 0.04M-solution of one of the three DL-phenylalanines.

The rate of formation of CO₂ with L-tyrosine was rapid; 132 μ l. CO₂ were evolved in the first 10 min. There was no CO₂ formation in any of the other flasks. This experiment shows that the chlorophenylalanines are not substrates of the bacterial decarboxylase.

In the second experiment a possible inhibitory action of the chlorophenylalanines on the bacterial enzyme was investigated. In this experiment the substrate was DL-*m*-hydroxyphenylalanine; this substance has the advantage over tyrosine of a greater solubility in water; also its affinity for the decarboxylase is less than that of tyrosine (Sloane-Stanley, 1949). The final concentration of DL-*m*-hydroxyphenylalanine and of each of the three DL-chlorophenylalanines was 0.0067M. It was found that the rate of CO₂ formation was not affected by the presence of any of the three chlorophenylalanines.

Oxidases

D-Amino-acid oxidase. All three chlorophenylalanines were oxidized by a preparation from pig kidney. In one experiment, in which 0.2 ml. of extract were used, the oxygen uptake in the first 9 min. of the experiment was with 0.028M-DL-methionine 147, with 0.014M-D-phenylalanine 34, with 0.028M-DL-*o*-chlorophenylalanine 13, with 0.028M-DL-*m*-chlorophenylalanine 82, and with 0.028M-DL-*p*-chlorophenylalanine 41 μ l. CO₂, respectively.

L-Amino-acid oxidase. In an experiment with 0.6 mg. of cobra venom in each flask, the oxygen uptakes in the 3–15 min. interval were with 0.028M-DL-methionine 52, with 0.028M-*o*-chlorophenylalanine 20, with 0.028M-*m*-chlorophenylalanine 20,

and with 0.028M-*p*-chlorophenylalanine 23 μ l. CO₂, respectively.

DISCUSSION

The experiments show that the three chlorophenylalanines are substrates of neither the mammalian nor the bacterial decarboxylases. The three amino-acids were found to be without a significant affinity for the two enzymes. These observations are of interest for the understanding of the substrate-enzyme relationship. They could be explained on the hypothesis that the enzyme-substrate reaction occurs through the phenolic hydroxyl groups of the analogous hydroxyphenylalanines, and that in this reaction the hydroxyl group cannot be replaced by chlorine.

The experiments give an illustration of the different degree of specificity of the amino-acid decarboxylases, as compared with the amino-acid oxidases. The three chlorophenylalanines are substrates of L-amino-acid oxidase and of D-amino-acid oxidase. Friedmann & Maase (1910) have shown that in the dog *p*-chlorophenylalanine as well as *p*-chlorophenylpyruvic acid are excreted as *p*-chlorophenylacetic acid in the urine. These observations find their explanation in the experiments here reported.

SUMMARY

1. The action of amino-acid decarboxylases and amino-acid oxidases on DL-*p*-, *m*- and *o*-chlorophenylalanine has been studied.
2. The mammalian 3:4-dihydroxyphenylalanine decarboxylase and the tyrosine decarboxylase of *Streptococcus faecalis* R do not act on these amino-acids; the compounds do not have any marked affinity for the two enzymes.
3. All three chlorophenylalanines are substrates of the D-amino-acid oxidase of pig kidney and of the L-amino-acid oxidase of cobra venom.

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