Microbiological Method for the Determination of L-Tryptophan

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ABSTRACT

SEBEK, Oldrich K. (The Upjohn Company, Kalamazoo, Mich.). Microbiological method for the determination of L-tryptophan. J. Bacteriol. 90:1026–1031. 1965.—The ability of Chromobacterium violaceum to utilize L-tryptophan for the synthesis of a purple pigment, violacein, served as a basis for the development of a quantitative estimation of this amino acid. The method consists of suspending washed colorless cells of the organism in an agar layer, placing a paper disc impregnated with a tryptophan solution on top of the layer, and allowing the system to incubate. As tryptophan diffuses into the agar, it is converted into violacein, and appears as a zone of striking purple color. Since the diameter of the zone is a function of the amount of tryptophan applied, the amino acid can be quantitatively estimated within the range of 10 to 320 µg per sample with 5.0% standard deviation. The method is fairly specific for free tryptophan, since only indole, indole-3-pyruvic acid, and, to a small degree, anthranilic acid interfere. Other amino acids, tissue homogenates, tryptophan in peptide linkage, or compounds related to this amino acid do not affect its determination. The bacterium does not utilize tryptophan for the synthesis of cellular material unless its growth has been initiated by another substrate.

Since the isolation of tryptophan in 1901, numerous methods for the determination of this amino acid have been designed and employed in the studies of the role which this compound plays in many forms of life. Although various chromatographic, mass spectrometric, and fluorimetric determinations are available at present, chemical estimations of this essential amino acid have been methods of choice in many such investigations. In principle, they are based on the ability of compounds containing indole nucleus to undergo chromogenic reactions with certain chemicals (Folin and Ciocalteau, 1927; Lugg, 1937; Eckert, 1943; Spies and Chambers, 1949; Dickman and Crockett, 1956; Mrskoš and Továrek, 1960; Inglis and Leaver, 1964). These chemical methods detect indole compounds rapidly and fairly well but have definite disadvantages. Since the final intensity of the color on which these tests are based and the rate at which it develops are affected by extraneous materials not related to tryptophan, they are not strictly specific for this amino acid.

A reliable way of determining tryptophan is by means of selected microorganisms. Such microorganisms not only respond to this amino acid fairly specifically, but detect exclusively its biologically active L-isomer (e.g., Shockman, 1963). One method employs tryptophanase of Escherichia coli which converts tryptophan rapidly and quantitatively to indole. When this indole in turn reacts with p-dimethylaminobenzaldehyde or p-dimethylaminocinnamaldehyde, the intensity of the color that develops is a function of the tryptophan present in the sample (Frank and DeMoss, 1957; Scott, 1961). Another estimation, the "analogue method," is based on the complete reactivation of growth of selected bacterial prototrophs (E. coli) which have been inhibited by 5-methyltryptophan or by other tryptophan antagonists (Mastroietro Cancellieri and Morpurgo, 1962). The phenylalanine test, which is required in the state of New York for the detection of phenylketonuria in infants, is based on the same ingenious principle (Guthrie, 1961). Recently, mouse fibroblasts were used in a similar system with only moderate success (Savichuck, Merriman, and Lockhart, 1964).

However, most of the microbiological methods that have been developed for the determination of this acid employ organisms for which tryptophan is an essential nutrient. Bacteria which have been used in such assays include selected strains of Clostridium perfringens (Boyd, Logan, and Tytell, 1948), Lactobacillus arabinosus (Henderson and Snell, 1948), L. plantarum (McGuire, Schiaffino, and Loy, 1960), Leuconostoc mesenteroides (Steele et al., 1949), and Streptococcus.
faecalis (Stokes et al., 1945). In principle, they represent an extension of the “auxanographic technique” described originally by Beijerinck (1891). On a heavily seeded solidified medium which lacks a substance specifically required for the growth of a given microorganism, no visible growth takes place. When, however, the missing substance is placed on the surface of such medium, it will diffuse around the application site and become available to the organism. Upon incubation, visible growth in the area of the diffused substance will identify this material as the factor essential for growth of the organism. (This technique is well established and has been used in various modifications not only for the detection and estimation of amino acids, but also of vitamins, peptides, components of nucleic acids, and growth factors. It has also been used for the detection of many artificially induced auxotrophs which have proved to be invaluable in elucidating various biosynthetic processes and in biochemical genetics. The detection of antibiotics, their antimicrobial spectra, and quantities may also be considered as an extension of Beijerinck’s technique, since it is based on the same principle, but measures growth inhibition instead of growth promotion.) Instead of a growth response, Beijerinck suggested other kinds of visible response such as luminescence or secretion of a pigment.

A striking example of the latter suggestion is the specific synthesis of a purple pigment violacein from L-tryptophan by Chromobacterium violaceum, which was reported by DeMoss and Evans (1959, 1960):

![Chemical structure of violacein](https://example.com/violacein.png)

My first use of this bacterium was for a different purpose. Since it carries out 5-hydroxylation of tryptophan (Mitoma, Weissbach, and Udenfriend, 1956), an important reaction which occurs in brain, it provided a simple and easily controlled system for the evaluation of the effect of various indole analogues on this reaction (Greig et al., 1961). In the course of this work, I found that this microorganism synthesizes violacein with unusual uniformity and reproducibility. Moreover, the amount of the pigment formed was found to be proportional to the amount of the L-tryptophan supplied. These characteristics were employed to develop quantitative estimation of this amino acid. Since to my knowledge this is the first instance where an organic substance is quantitatively measured in terms of a biologically produced pigment, the method is here described.

**Materials and Methods**

C. violaceum ATCC 553 was obtained from R. D. DeMoss, University of Illinois. It was carried on slants containing 0.5% each of peptone and yeast extract (both from Difco), adjusted to pH 7.0 to 7.4, and solidified with 1.5% agar. It was transferred at biweekly intervals, and maintained at room (23 to 26 C) temperature. The amino acid requirements of the bacterium were established by sequential elimination of individual amino acids from amino acid mixtures which were dissolved in an inorganic salt solution containing 20 mg of MgCl2, 60 mg of K2HPO4, 5 mg of K2SO4, and 0.1 mg of FeSO4 in 100 ml of distilled water. The mixtures were incubated with washed and starved cells, and were incubated in shaken flasks at room temperature for 1 to 5 days. Growth was measured by determining the dry (110 C) cell weight of the samples taken at different times during the experiments. Some of the violacein synthesized by the cells was also released into the medium. For this reason, the clear supernatants were combined with the pyridine extracts of the centrifuged cells, and the amount of violacein was then determined in the pooled samples from a previously prepared standard curve. The curve related the transmittance of the color to micrograms of violacein per milliliter at 570 m,u, which was the maximal absorbance of an authentic sample of our violacein preparation.

The actual determination of tryptophan was carried out by inoculating the cells into 100 ml of the above peptone-yeast extract medium containing 0.1 to 0.4% glucose in 250-ml Erlenmeyer flasks, and incubating them overnight at room temperature and at 230 to 300 rev/min on a New Brunswick shaker. The colorless or faintly purple cells from 40 to 80 ml of this medium were removed by centrifugation and washed with sterile water; the cells were then suspended in 100 ml of 2% agar containing 20 mg of MgCl2, 60 mg of K2HPO4, 5 mg of K2SO4, 0.1 mg of FeSO4, adjusted to pH 7.2, and cooled to 42 to 44 C. Samples (10 ml) of this medium were then rapidly dispensed into petri plates. After solidification, small filter discs (0.6-cm diameter) were placed on the agar surface, and 0.02 ml of solutions containing L-tryptophan,
either alone or in mixtures with other components, was pipetted on the discs. In like manner, solutions of 10 to 320 μg of L-tryptophan per 0.02 ml were prepared as standards. The plates were then incubated at room temperature overnight. As tryptophan diffused into the agar, it was converted by the bacteria into violacein, which was easily detected as purple zones around the discs (Fig. 1). The diameter of the zone is a function of the tryptophan concentration and forms a straight line when these values are plotted on semilogarithmetic paper within 10 to 320 μg per disc (Fig. 2). The amounts of the amino acid are then easily interpolated from the standard. The zone measurements were made with the aid of Quebec bacteria colony counter except in the experiments designed for statistical evaluation of the method, in which case a Fischer-Lilly antibiotic zone reader was used.

RESULTS AND DISCUSSION

Studies of the amino acid requirements showed that this bacterium grew in a simple chemically defined medium which consisted of inorganic salts supplemented only with L-alanine in 0.1 to 1.0% concentrations. Glutamate and glutamine were utilized equally well, whereas L-phenylalanine, L-tyrosine, and L-histidine supported growth to a smaller extent. L-Tryptophan was not utilized for growth when added to the medium as the only carbon and nitrogen source. However, after the growth had been initiated by any of the above substrates, it was used both for growth and for the violacin synthesis as illustrated in Fig. 3. No growth took place in the inorganic salts medium supplemented with 50 to 1,000 mg of tryptophan per 100 ml. When 500 mg of alanine per 100 ml were supplied as the only organic substrate, 50 mg of dry cells containing 1 mg of violacine per 100 ml were produced in 110 hr. However, when tryptophan was added to this alanine base in the 50 to 250 mg per 100 ml concentrations, the yields of the dry cells increased to 71 to 80 mg, and of violacine to 6.1 to 7.0 mg per 100 ml in the same incubation period. Higher tryptophan concentrations inhibited the growth to some degree and also retarded the violacin synthesis.

The cells required for the tryptophan deter-

![Fig. 1. Violacin formation from L-tryptophan by washed cells of Chromobacterium violaceum suspended in an agar medium. A 100-μg amount of tryptophan solution was pipetted on the filter disc and the plate was incubated for 16 hr at room temperature.](image)

![Fig. 2. Relationship between the diameter of the pigmented zones and L-tryptophan concentration.](image)

![Fig. 3. Utilization of DL-alanine and L-tryptophan for growth and violacin synthesis. Symbols: 0.5% DL-alanine alone (□) and with increasing amounts of L-tryptophan: 0.05% (●), 0.1% (△), 0.25% (○), 0.5% (□), 0.75% (●), and 1.0% (▽). No growth in L-tryptophan. The bacteria were grown in 50 ml of inorganic salts containing the amino acids as indicated per 250-ml Erlenmeyer flask, aerated (New Brunswick shaker, 260 rev/min) at 28 C.](image)
mination by this method have to be essentially colorless. As illustrated by the experiment summarized in Fig. 4, they started synthesizing the pigment only after they had reached approximately the middle of logarithmic growth. Depending on the amount of the inoculum (0.07 to 16.6 mg of dry cells per 100 ml of medium), this occurred in the 9th to 15th hour of incubation. Within the subsequent 4 to 8 hr, the population developed a faintly purple color which in time turned deep purple and therefore became undesirable for the test. In a detailed evaluation of the method, a 15- to 18-hr incubation period was found to produce cells suitable for the assay, especially when the growth medium had been supplemented with 0.4 to 1.0% glucose, which further delayed the onset of the violacein formation. In the media devoid of tryptophan, violacein was produced only after several days of incubation of fully grown cells. In such cells, endogenous tryptophan was no longer needed for growth and was shunted to the pigment synthesis. The amount of cells used for the incorporation into the agar has to be the same in each series of determinations, since heavier suspensions produce smaller (but more deeply pigmented) zones. This relationship is illustrated in Table 1. Cells from 40 to 60 ml of the medium, washed and suspended in 100 ml of agar, gave satisfactory pigmentation for the range of the tryptophan concentrations as indicated in Fig. 2.

As also seen in Fig. 2, a straight-line relationship exists between the zone diameter and the tryptophan concentration. A statistical analysis of this assay, which was carried out as a randomized block design with six concentrations on each of nine different plates, showed that the standard deviation (a) for the scatter of the concentration means about the straight line was 0.37 mm. The slope of the standard curve (b) was 16.9 mm per 10-fold increase in concentration. Hence the precision index (λ) = = 0.022. The determination of unknown concentrations had, therefore, a standard deviation of 230.3 × (0.022) \( \sqrt{1 = 1/5} = 5.6\% \). Although as little as 10 to 30 \( \mu \)g of the amino acid per disc could be detected, the zones corresponding to these low concentrations were frequently hazy. It is therefore recommended that 50 \( \mu \)g of tryptophan per disc be the lower limit in routine estimation.

In the recovery experiments, tryptophan was added to several homogenized rat tissues (lung, spleen, heart, liver, kidney, brain, skeletal muscle) and to heavy suspensions of C. violaceum, in the 5 mg/ml final concentration. All the preparations were then inactivated by heat (15 min at 121 C), and the centrifuged supernatant fluids were assayed for tryptophan after 1, 2, and 3 days of storage at 5 C. In all instances, 93 to 101% tryptophan recovery was obtained. In addition to these materials, a number of substances of varying complexity were tested and were found not to interfere with tryptophan determination: (i) human serum, lysozyme, lactalbumin, gelatin, casein, and its pancreatic (Casitone) and acid hydrolytic digests (Casamino Acids); (ii)

![Graph of pigmented and non-pigmented cells](image)

**Fig. 4.** Relationship between the age of Chromobacterium violaceum and the formation of violacein. Inoculum (dry cells/100 ml): 0.07 mg (X) 0.33 mg (○), 1.66 mg (△), 3.52 mg (▽), 6.64 mg (■), 18.80 mg (▲). The bacteria were grown in 50 ml of a medium containing 0.5% each of peptone and yeast extract (pH 7.2) per 250-ml Erlenmeyer flask, aerated (New Brunswick shaker, 280 rev/min) at 28 C.

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<th>Vol from which the cells were obtained</th>
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<td>ml</td>
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* Cells (3.07 mg, dry weight/ml) from volumes as indicated (glucose-peptone-yeast extract medium) incubated for 16 hr at 28 C, washed, and suspended in the agar; 80 \( \mu \)g of L-tryptophan per disc applied and incubated overnight.
essential and nonessential amino acids; and (iii) compounds related to L-tryptophan (its d-isomer, 5-hydroxy-L-tryptophan, tryptamine, 5-hydroxytryptamine, 5-methyltryptamine, 5-hydroxyindole, 5-bromoindole, 5-bromoindole-3-carboxaldehyde, gramine, indole-3-propionic acid, indole-3-acetic acid, 5-hydroxyindole-3-acetic acid, skatole, isatin, 3-hydroxyanthranilic and nicotinic acids, L-kynurenine, oxindole, and indoxyl acetate, glycyl-L-tryptophan, acetyl-D,L-tryptophan). [Indoxyl acetate was rapidly hydrolyzed and subsequently oxidatively condensed into indigo, a light-fast, brilliant deep-blue pigment, easily distinguishable from violacein. This transformation, which demonstrates that this bacterium also synthesizes an esterase, was suggested for histochemical localization of this enzyme in animal tissues (Barnett and Seligman, 1951; Holt, 1952.).] In addition, tryptophan determination was not interfered with by α-ethyltryptamine, an established monoamine oxidase inhibitor, and of many of its analogues, which were evaluated in this system (Greig et al., 1961).

Rapid formation of violacein from tryptophan is fairly specific, since only indole and indole-3-pyruvic acid were also found to form this pigment, and thus to interfere with this tryptophan determination. Another compound, anthranilic acid, also acted in this way, but to a negligible extent when evaluated in this (agar plate) system, and it did not produce any pigment when tested in the liquid medium (Sebek and Jäger, 1962a). These compounds in the sample may be rapidly separated by any available method, such as by thin-layer chromatography adapted from the paper chromatography technique described by Fink, Cline, and Fink (1963), followed by extraction directly from the plate. If indole is the only interfering substance, it may be easily removed from the sample with solvent extraction at an alkaline pH. Interference by these compounds is not completely unexpected, since they are involved in the metabolism of tryptophan. Anthranilic acid is its established precursor, and indolepyruvic acid is formed by this bacterium from tryptophan by transamination with α-ketoglutaric acid (DeMoss, 1957), whereas the reverse has been demonstrated in several other microorganisms (Aida et al., 1958; Asai et al., 1962).

Indole itself was found to be formed from tryptophan by this organism (Sebek and Jäger, 1962a, b).

The bacterium was received in our laboratories in 1957. It has since been transferred in biweekly intervals on glucose-peptone-yeast extract-agar, maintained at 23 to 28 C and never refrigerated. Under these conditions, it had simple amino acid growth requirements as described above (cf. DeMoss and Happel, 1959).

Although not observed by others to be a characteristic of this genus (Sneath, 1956; De Ley, 1964; DeMoss, personal communication), this strain exhibited practically no color variation when plated out on agar medium of the above composition for resolation. This stability is another desirable feature of this strain for the described determination.

Acknowledgments

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Literature Cited


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