conversion factor to correlate the absorbance values obtained with the chromophore substrate to a number of nmoles bonds hydrolyzed per min can be obtained by a parallel assay of a standard α-amylase preparation of the same origin calibrated in the same units of activity (nmol/min^{-1} at 37 °C).

KOSHLAND[5] considers β-amylase to be an example of the 'induced fit' concept, which accounts for the specificity of this enzyme in producing exclusively maltose, and the failure, as opposed to α-amylase, to bypass branch points. In this model, an adaption of the conformation of β-amylase with regard to the substrate is induced: one of the non-reducing ends of the substrate penetrates into the enzyme molecule, changing its conformation in such a way that the penultimate glycosidic linkage can be hydrolyzed. This mechanism of interaction between the starch substrate and β-amylase makes conceivable also the inability of the enzyme to attack an α-1,4 glucan chain, partial substituted in carbonatom 6.

As substituent the Cibachron blue F3GA proved to be indicated for this purpose. It can be coupled in a single way[6,7] to a polyglucan chain by an ether bond.

If starch is the substrate, the primary hydroxyl function of carbon atom 6 of the glucose monomers will be attacked by a nucleophilic substitution. The two other secondary hydroxyl functions have not only a less acidic character, but occupy also in the helix of amylose postions which, for stereochemical reasons, are difficult to substitute. The hydroxyl group on carbon atom 6, on the other hand, extends above the plane of the pyranose ring and is also situated on the outside of the amylose helix. Since the diameter of the Cibachron F3GA molecule is not only larger than that of the amylose-helix (13 Å), but also larger than the distance between 2 neighbouring helical turns (8 Å), we may assume that these chromogenic molecules are situated on the outside of the helix.

Zusammenfassung. Bei Anwendung eines Stärkesubstrates in Verbindung mit einem im Kohlenstoffatom 6 kovalent gebundenen Farbstoffmolekül gelingt die differentielle Messung der α- und β-Amylase Aktivität im Reaktions-medium.

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Thin Layer Chromatography Methods for Detecting Hippurate Hydrolase Activity among Various Bacteria (*Pseudomonas*, *Bacillus*, *Enterobacteriaceae*)

Hydrolysis of the herbivoric excretion product, hippuric acid (N-benzoyl-glycine), is catalyzed in microorganisms by the specific enzyme hippurate hydrolase[1] EC 3.5.1. x:

$$\text{hippuric acid} \xrightarrow[\text{hydrolase}]{\text{hippurate}} \text{benzoic acid} + \text{glycine}$$

So far, the hydrolysis of hippurate by microorganisms was proved either by growth in a synthetic sodium hippurate broth[2], or by precipitation of benzoic acid with ferric chloride, by crystal formation with sulfuric acid[3,4], by the use of a pyridine-copper sulphate reagent[5] or by a shift of the pH in a suitable hippurate agar[6] into the alkaline range. The reliability of these various methods, however, is quite doubtful, as has been shown recently by comparative studies[7,8].

While characterizing an unidentified *Pseudomonas* species, the following TLC procedures were found very convenient and reliable in detecting hippurate hydrolysis among various, nutritionally versatile bacteria.

Cultivation and medium. Erlenmeyer flasks (250 ml), containing 50 ml of a complex sodium hippurate-(10 g/l)-glucose (1.0 g/l)-broth[8], were inoculated and orbit shaken for 3 weeks at 28 °C.

Chromatography. From each flask, 1 ml samples were taken, acidified with 1 ml 1 *N* H_2SO_4, mixed with 1 ml chloroform to extract benzoic acid and the aqueous supernatant (containing the cells) removed by vacuum suction. The chloroform extract was dried with Na_2SO_4 and 4 µl amounts applied to the following precoated thin

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Table I. Specific colorization of hippuric- and/or benzoic acid by various sprays recommended for organic acids

Spray-type	Hippuric acid	Benzoic acid	Background
1. Ferric cyanide/ferric chloride[10]	—	white	blue
2. $CuSO_4$ solution[11]	white	white	blue-green
3. Glucose/aniline[12]	dark-brown	dark-brown	yellow-brown
4. Bromcresolgreen/bromphenolblue[13]	blue	orange	grey-blue
5. $FeSO_4/H_2O_2/MnSO_4$[14]	dark-brown	dark-brown	white

Table II. Detection of hippurate hydrolase by identification of benzoic acid

Organisms tested	benzoic acid shown by UV-viewing (Rf = 0,15)	spray number 1, 2, 3, 4 or 5
Pseudomonas mendocina ATCC 25411	–	–
Ps. stutzeri ATCC 17588	–	–
Ps. stanieri ATCC 17591	–	–
Ps. testosteroni ATCC 11996	–	–
Ps. fluorescens CCM 2115	–	–
Ps. aeruginosa CCM 1960	–	–
Ps. saccharophilia CCM 1980	+	+
Pseudomonas sp. (No. 15, 16, 17, 19)	+	+
Enterobacter aerogenes	–	–
Serratia marcescens	–	–
Proteus vulgaris	+	+
Escherichia coli B	–	–
Bacillus brevis SMG 298	+	+
B. macerans ATCC 8244	–	–
B. psychrosaccharolyticus ATCC 23296	–	–
B. cereus var. mycoides CCM 48	–	–
B. firmus CCM 37	+	+
B. lentus CCM 35	–	–
B. pumilus ATCC 7061	+	+

ATCC, American Type Culture Collection; SMG, Sammlung für Mikroorganismen, Göttingen; CCM, Chechoslovak Collection of Microorganisms, Brno.

layer (0.1 mm) plates: a) Cellulose TLC aluminium sheets (20 × 20 cm), without fluorescence indicator (Merck AG, Darmstadt, Germany, article number 5552) or b) SIF silica gel sheets (9.5 × 18 cm) with fluorescence indicator (Riedel-de-Haen, Hannover, Germany, article number 37350).

As a reference, on each sheet 4 µl amounts of a 1 ml chloroform-extracted sample of an aqueous benzoic acid solution (6.0 g/l) were spotted. Ascending one-dimensional chromatograms were run with a solvent system of *n*-hexane/acetic acid (96/4) for 30 min, air-dried and the fluorescence indicator treated plates observed under UV-light for deep-purple benzoic acid spots. The cellulose sheets were sprayed either with *p*-diaminobenzaldehyd acetic-acidanhydride[9] (specific red-orange spots with hippuric acid) or with one of the reagents listed in Table I.

Results. In Table I, the detection of benzoic- and hippuric acid by several sprays is given. With the solvent system used, hippuric acid remained at the point of spotting, whereas benzoic acid moved differently on silica gel sheets (Rf = 0.15) and on cellulose (Rf = 0.72). The unequivocal evidence as well as the complete correlation of results (Table II) between both UV-viewing and the various sprays warrant a clear-cut detection of hippurate hydrolysis under the conditions given.

Among the sprays tested, the $FeSO_4/H_2O_2/MnSO_4$-reagent of GOSSLÉ and SREBRNIK-FRISZMAN[14] proved to be most sensitive on benzoic acid. Therefore, if a great number of organisms are to be compared for their ability to hydrolize hippuric acid, either precoated silica gel sheets with fluorescence indicator or cellulose aluminium sheets sprayed with $FeSO_4/H_2O_2/MnSO_4$ reagent are recommended[15].

Zusammenfassung. Zum Nachweis von Hippurat Hydrolase bei Mikroorganismen wird die Dünnschichtchromatographie auf Silicagel-Fluoreszens-Indikator Fertigplatten oder auf Cellulose-Alufolien mit Hexan/Essigsäure (96/4) als Laufmittel zur Trennung von Benzoe- und Hippursäure empfohlen. Benzoesäure gibt auf DC-Karten SIF Kieselgel mit Fluoreszensindikator unter UV violette Flecken, während sie auf DC-Alufolien (Cellulose) mit $FeSO_4/H_2O_2/MnSO_4$-Reagenz[14] empfindliche, dunkelbraune Flecken gibt (Rf = 0.72). Hippursäure bleibt mit Hexan/Essigsäure als Laufmittel am Auftragungsort zurück. Beide Nachweisverfahren, geprüft an verschiedenen Bakterien, stimmen vollständig in den Ergebnissen überein (Table II). Für Routineuntersuchungen genügt infolgedessen eines der beiden Verfahren.

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