be mycoplasm-like organisms or products of their metabolism, or both.

If these observations are validated, then they could provide a simple method for preliminary screening of infected tissues, particularly over large areas.

Résumé. Une méthode simple est décrite pour l'étude radioscopique des tissus du bois de Santal (*Santalum album* L.) infectés par un organisme du type dit mycoplasme. Les techniques comprennent au préalable l'emploi de colorants Giemsa et Dienes, précédant l'étude microscopique. Des organismes du type dit mycoplasme et (ou) des inclusions cytoplasmiques d'origne mycoplasmique ont été localisées dans les tissus contaminés du bois de Santal, surtout dans les régions du xylème et du phloème.

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The Amylolysis of a Substituted Starch Substrate

Although a large number of procedures for the assay of α- or β-amylase are reported in the literature, the separate measurement of the activities of these enzymes in the same reaction medium still poses a problem in investigations dealing with the occurrence of α- and β-amylase isozymes in germinating seeds or when studying applications in both industrial fermentation processes and in food and pharmaceutical industry. Preliminary experiments based on a controlled selective denaturation of one of the enzymatic partners in the reaction mixture have not given us satisfying results.

The difference of rheology [1] during the α- or β-amylolysis does not allow discrimination between α- and β-amylolytic activities, since viscosimetric measurements follow the variation of the weight average degree of polymerization of the substrate and the reaction products, while the expression of the hydrolytic activity is a function of the numerical average degree of polymerization. It is obvious that the variation of these two parameters during the endohydrolysis by α-amylase or the exohydrolytic attack by β-amylase does not proceed in the same manner, so that it is difficult to establish in both cases a relationship between a drop in viscosity and the hydrolase-activity.

We found, however, that by coupling, for instance, a chromogenic molecule to starch, the exohydrolytic action of β-amylase on this substrate is inhibited, while these substituents on the helixes of the substrate only function as accidental barriers for α-amylase and do not hinder the progressing endohydrolytic attack. To find a suited substituent was not so difficult, since chromogenic substances which can be bound covalently with the glucose monomers, were developed for the textile-industry. For this purpose the Cibachron Blue F3GA (Ciba), a chromogenic product of the monochlorotriazine type, proved to be indicated.

Material and methods. Crystalline α-amylase from hog pancreas and β-amylase from sweet potatoes are prepared by the Worthington Biochemical Corporation. Dilutions are made with a solution containing 0.1% ovalbumin (Sigma Grade V), 16% NaCl and 0.05% $CaCl_2.2H_2O$. The measurement of the total α- and β-amylolytic activity is performed by means of a reductometric method [2], with starch after Zulkowsky (Merck) as substrate and 3,5-dinitrosalicylic acid as oxydizing agent. The assay of the α-amylase activity using a chromophore substrate (Phadebas, Pharmacia) is conducted by the colorimetric procedure [3] or by the plate diffusion technique [4] described by CEŠKA.

Results and discussion. The influence of a substitution starch substrate on the exo- and endo-amylolytic attack can very well be evaluated semiquantitatively with the help of a plate diffusion technique by incorporation in the agargel of a suitable chromophore (Cibachron F3GA) starch preparation. We find that only α-amylase, due to its endoamylolytic activity, produces a well defined circular transparent digestion zone, while the presence of β-amylase, even in high amounts (90% w/w), does not give a visible effect. We could also differentiate the α- and β-amylolytic activities quantitatively in the same preparation (Table). The total α- and β-amylase activity, in nmoles glucosidic bonds hydrolysed per min, is measured with a reductometric method, while the specific colorimetric dosage of the α-amylase activity is performed by means of an insoluble chromogen coupled substrate. The

[1] S. SCHARPÉ and A. LAUWERS, Farm. Tijdschr. België *48*, 354 (1971).
[2] S. SCHARPÉ, Clin. chim. Acta *37*, 301 (1972).
[3] M. CEŠKA, K. BIRATH and B. BROWN, Clin. chim. Acta *26*, 437 (1969).
[4] M. CEŠKA, Eur. J. Biochem. *22*, 186 (1971).

Differentiation of α- and β-amylolytic action

Enzyme per assay (ng)		α-amylase activity (chromophore substr.)	Total α- and β-amylase activity (reductometric)	β-amylase activity
α-amylase	β-amylase	(A_{620} converted to nmol/min^{-1})	(nmol/min^{-1})	(nmol/min^{-1})
0	9	0	27	27
18	9	13	40	27
36	9	26	53	27
54	9	39	66	27

To evaluate the α-amylase activity in nmol/min^{-1}, the absorbance values measured using the chromophore substrate, are multiplied by 0.23.

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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[5] J. THOMA and D. KOSHLAND, J. Am. chem. Soc. *82*, 3329 (1960).
[6] H. RINDERKNECHT, P. WILDING and B. HAVERBACK, Experientia *23*, 805 (1967).
[7] M. CESKA, E. HULTMAN and E. INGELMAN, Experientia *25*, 555 (1969).

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

[1] W. RISCHKA and M. RÖHR, Zentbl. Bakt. ParasitKde *127*, II, 222 (1972).
[2] A. A. HAJNA and S. R. DAMON, Am. J. Hyg. *19*, 545 (1934).
[3] G. H. G. DAVIS, J. gen. Microbiol. *13*, 481 (1955).
[4] R. E. GORDON, J. gen. Microbiol. *43*, *329* (1966).
[5] E. MUNSCH-PETERSEN, Bull. Coun. Scient. ind. Res. Melb. *34*, 96 (1940).
[6] M. L. THIRST, J. gen. Microbiol. *17*, 390 (1957).
[7] P. ZIEGLER and H. J. KUTZNER, Z. allg. Mikrobiol., *13*, 265 (1973).
[8] J. C. G. OTTOW, J. appl. Bact., 1973, in press.

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