Table 1. Effects of 25-hydroxyvitamin-D on insulin release from leucine-stimulated normal and vitamin D deficient pancreatic islets incubated 90 min at 37 °C

Treatment	Insulin release (μunit/islet)
Normal	
Control, 0 ng/ml	59 ± 3[a]
25-Hydroxyvitamin-D, 10 ng/ml	56 ± 2
25-Hydroxyvitamin-D, 40 ng/ml	63 ± 3
25-Hydroxyvitamin-D, 100 ng/ml	57 ± 2
Vitamin D deficient	
Control, 0 ng/ml	54 ± 2
25-Hydroxyvitamin-D, 10 ng/ml	43 ± 2
25-Hydroxyvitamin-D, 40 ng/ml	48 ± 2
25-Hydroxyvitamin-D, 100 ng/ml	45 ± 2

[a] Mean ± SE of the mean of 4 observations in duplicate.

Table 2. Effects of 1,25-dihydroxyvitamin-D on insulin release from glucose-stimulated normal and vitamin D deficient pancreatic islets incubated 60 min at 37 °C

Treatment	Insulin release (μunit/islet)
Normal	
Control, 0 ng/ml	46 ± 2[a]
1,25-Dihydroxyvitamin-D, 100 ng/ml	42 ± 3
Vitamin D deficient	
Control, 0 ng/ml	44 ± 3
1,25-Dihydroxyvitamin-D, 100 ng/ml	44 ± 4

[a] Mean ± SE of the mean of 6 observations in duplicate.

Young vitamin D deficient rats were purchased from Harlan Sprague-Dawley, Indianapolis, IN. Adult vitamin D deficient rats were obtained from Dr H. F. DeLuca, University of Wisconsin, Madison, WI. Animals were killed immediately upon arrival and the pancreas removed for islets isolation.

Ten intact islets, capable of insulin release, were handpicked twice, placed in ice-cold Eagles' solution and stored on ice for 30 min prior to addition of the secretagogues (10 mM L-leucine and 10 mM L-glutamine or 200 mg/dl glucose) and the test compounds. 25-Hydroxy-vitamin-D and 1,25-dihydroxyvitamin-D were gifts from Dr J. C. Babcock, The UpJohn Co., Kalamazoo, MI, and Dr M. R. Uskokovic, Hoffmann-La Roche, Nutley, NJ, respectively. The compounds were dissolved in 95% ethanol and subsequently diluted in the Eagles' incubation medium to give a final ethanol concentration of 0.1% which did not interfere with insulin release from the islets (data not shown). The islets and test compounds were incubated 60–90 min at 37 °C in a Dubinoff shaker. The reaction was stopped and the insulin released was measured as previously reported[4].

A two-tailed Student t-test was used to ascertain levels of significance[5]. Probability values of 0.05 or less were considered significant.

Results and discussion. Table 1 shows the effect of vitamin D deficiency on insulin release from leucine-stimulated pancreatic islets of young Sprague-Dawley rats (100–150 g). Although there were observable differences between the two treatments (vitamin D deficient rats had less fat, showed a more friable skin, and a more easily digested pancreas by collagenase), no differences were observed for the release of insulin from the isolated pancreatic islets. No differences were found in insulin release upon the addition of 25-hydroxyvitamin-D to the islets of normal or vitamin D deficient rats. Similar results were recorded for glucose-stimulated pancreatic islets of adult (200–400 g) vitamin D deficient and normal rats (table 2). No differences in insulin release from glucose stimulation were found between the two treatments. The addition of 1,25-dihydroxyvitamin-D to the islets of either group did not affect insulin release; it remained the same.

Therefore, it may be concluded that vitamin D deficiency, in these tests, did not alter the release of insulin from isolated pancreatic islets nor was there a direct in vitro effect of 25-hydroxyvitamin-D or of 1,25-hydroxyvitamin-D on the release of insulin by the isolated pancreatic islets. We thus conclude that the previous reports of vitamin D effect on insulin secretion[1–3] must be related to synthesis and not to release from the pancreatic islet.

1 Norman, A. W., Frankel, B. J., Heldt, A. M., and Grodsky, G. M., Science *209* (1980) 823.
2 Kadowski, S., and Norman, A. W., J. clin. Invest. *73* (1984) 759.
3 Clark, S. A., Stumpf, W. E., and Sar, M., Diabetes *30* (1981) 382.
4 Rogers, K. S., and Evangelista, S. J., Proc. Soc. exp. Biol. Med. *178* (1985) 275.
5 Edwards, A. L., in: Statistical Analysis, p. 127. Rinehart, New York 1958.

0014-4754/86/11/121253-02$1.50 + 0.20/0

Anaerobic biodegradation of m-, o-, p-hydroxycinnamic acid by an adapted microbial consortium

V. Andreoni, M. Nali*, B. Rindone*, St. Tollari*, V. Treccani and M. Villa

*Institute of Agrarian Microbiology, University of Milan, Via Celoria 2, I-20133 Milan (Italy) and *Department of Organic and Industrial Chemistry, University of Milan, Via Venezian 21, I-20133 Milan (Italy), 22 July 1985*

Summary. The degradation of ortho-, meta- and para-hydroxycinnamic acid by an acclimated consortium (or 'mixed bacterial culture') was investigated. The biodegradation was evaluated by monitoring substrate disappearance. The relative rates of consumption were in the order: para > meta > > ortho. Only in the catabolism of meta- and para-hydroxycinnamic acid, the demolition of the side chain by the loss of a C_1 unit is involved. The utilization of para-hydroxycinnamic acid by the consortium occurred rapidly and completely within a 38-day incubation.

Key words. Anaerobic metabolism; acclimated consortium; hydroxycinnamic acid isomers.

In the context of studies on the anaerobic degradation of phenolic acids, which are widespread in nature, Andreoni et al.[1] described a consortium (or 'mixed bacterial culture') that grew on caffeic acid as the only carbon and energy source. It was also able to utilize ferulic acid, cinnamic acid, protochatecuic acid, vanillic acid, m- and p-hydroxycinnamic acid. Nali et al.[2] gave evidence that three reactions are involved in the early stages of the anaerobic catabolism of some phenylpropenoic acids; the

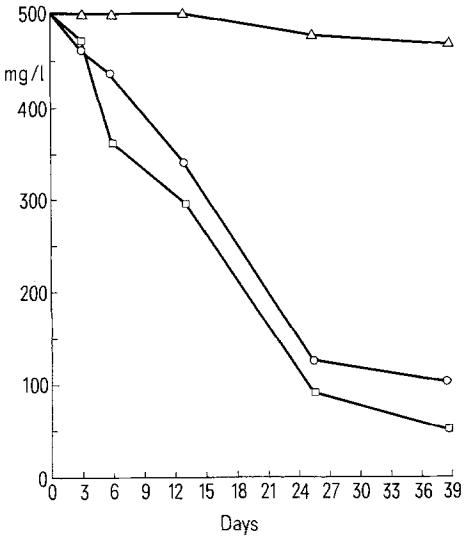


Figure 1. Degradation rates of hydroxycinnamic acids expressed as concentration of total recovered material versus time in cultures fed with ortho-, –△–; meta-, –○–; and para-, –□–, hydroxycinnamic acid.

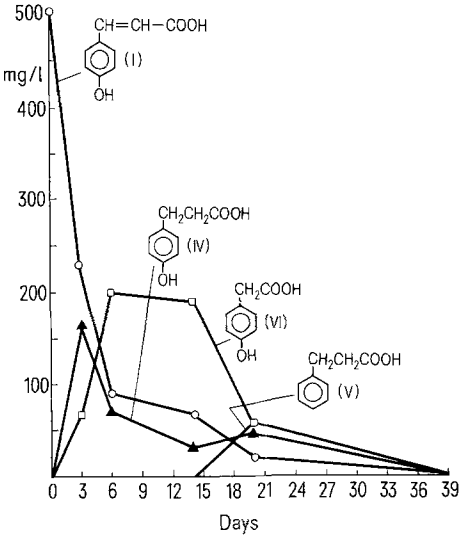


Figure 2. Time course of the anaerobic metabolism of para-hydroxycinnamic acid by acclimated cultures.

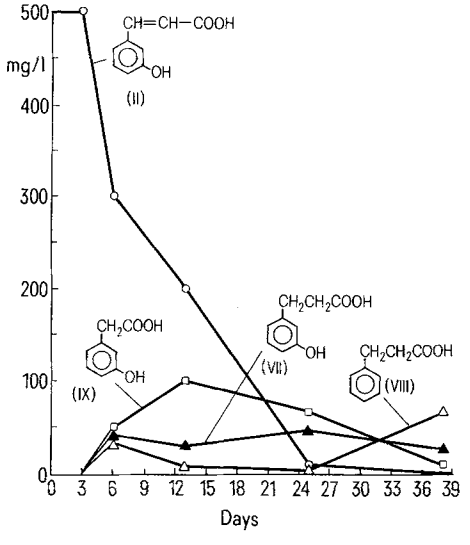


Figure 3. Time course of the anaerobic metabolism of meta-hydroxycinnamic acid by acclimated cultures.

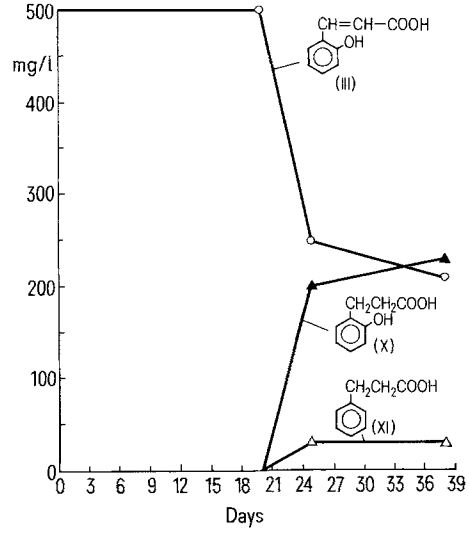


Figure 4. Time course of the anaerobic metabolism of ortho-hydroxycinnamic acid by acclimated cultures.

reduction of the double bond of the side chain, the dehydroxylation of the aromatic ring mainly from the para position and the demolition of the side chain by the loss of a C_1 unit.

The purpose of this study is to confirm the positional selectivity in the dehydroxylation reaction, which was noted to depend on the position of the hydroxyl group attached to the aromatic ring. This was performed by examining the anaerobic fate of the ortho-, meta- and para isomers of hydroxycinnamic acid.

Cultures previously described[2] and acclimated initially to p-hydroxycinnamic acid were tested against the two remaining isomers.

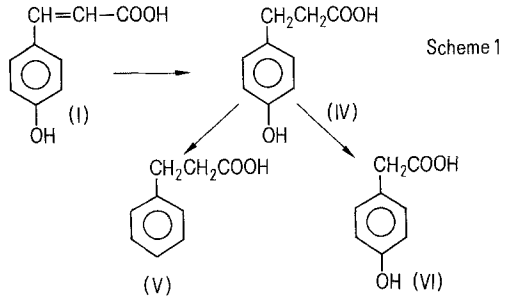
Material and methods. Chemicals. p-, m- and o-hydroxycinnamic acid and all other chemicals were of the highest purity commercially available. Gas mixtures were supplied by S.I.A.D., Bergamo, Italy.

Analytical determinations. Spectrophotometric analyses were performed using a Beckman 24/25 spectrophotometer. GLC-MS spectra were obtained with a Varian Mat 112 instrument equipped with a 1.5-m glass column (SE 30 3% on chromosorb W; oven temperature 70–220 °C, 10 °C/min; injection temperature 250 °C; carrier gas helium 30 ml/min.

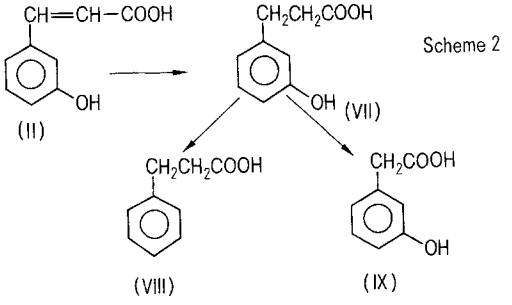
Quantitative analyses were performed using a Varian Vista 6000 gas chromatograph equipped with a 50-cm stainless steel column (OV 101 5%, oven temp. 150–220 °C, 35 °C/min, inj. temp. 250 °C, flame ionization detector temp. 250 °C, carrier gas nitrogen 25 ml/min). Peak areas were integrated with a Varian 4270 integrator.

The reaction products were detected by GLC-MS after diazomethane treatment of the crude extracts at pH = 2 and were identified by comparison with authentic specimens.

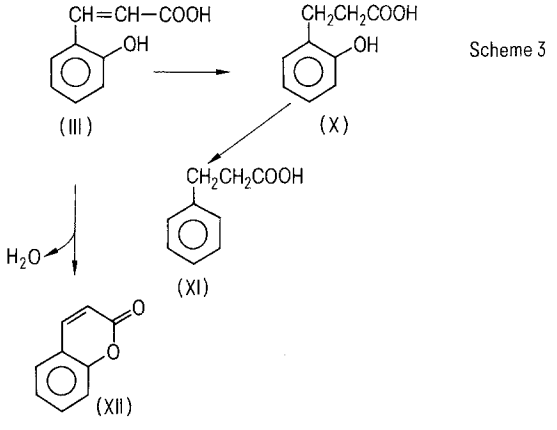
Microorganisms. Cultural and anaerobic conditions were those previously described[2]. The consortium[1] was acclimated to para-, meta- and ortho-hydroxycinnamic acid. The cultures were then maintained by a periodic transfer in 250-ml rubber stoppered bottles filled with freshly prereduced mineral medium (FW) added with 0.05% (w/v) hydroxycinnamic acid isomers as the



Scheme 1. The metabolic pathway in the degradation of para-hydroxycinnamic acid.



Scheme 2. The metabolic pathway in the degradation of meta-hydroxycinnamic acid.



Scheme 3. The metabolic pathway in the degradation of ortho-hydroxycinnamic acid.

only organic carbon source, and incubated at 35 °C under an atmosphere of hydrogen and carbon dioxide 8:2.

Results. p-Hydroxycinnamic acid (1) degradation. The consortium adapted on p-hydroxycinnamic acid as the sole carbon and energy source degrades freshly added p-hydroxycinnamic acid 0.05% without any lag period. Samples withdrawn by syringe after 0, 2, 4, 6, 12, 20, 38 days were analyzed spectrophotometrically for residual aromatic compounds. Almost complete consumption of the substrate was observed after 20 days of incubation (fig. 1). Intermediates in this transformation were p-hydroxyphenylpropionic acid (**4**), phenylpropionic acid (**5**) and p-hydroxyphenylacetic acid (**6**) (scheme 1).

This metabolic pathway is in complete accord with the anaerobic catabolism pattern already observed with some other hydroxycinnamic acids[2].

In order to determine the amounts of all the products involved in the anaerobic metabolism of this compound with time, one 500-ml rubber stoppered bottle filled with mineral medium containing 0.05% (w/v) p-hydroxycinnamic acid was inoculated with a 10% preculture. 50-ml aliquots were removed from the anaerobic culture at successive incubation times and extracted with ethyl acetate at pH = 2. The quantitative analyses were performed by GLC after diazomethane treatment of the crude extracts. The amount of each compound is given in figure 2. Utilization of p-hydroxycinnamic acid by the cultures appears to occur rapidly and without a lag period.

m-Hydroxycinnamic acid (2) degradation. The same consortium was transferred by successive passages to m-hydroxycinnamic acid 0.05%. Results similar to those with the para isomer were observed. In fact, m-hydroxyphenylpropionic acid (**7**), phenylpropionic acid (**8**) and m-hydroxyphenylacetic acid (**9**) were detected (scheme 2). From the quantitative data (fig. 3) a short lag period before the start of the transformation is noted. After long incubation times (38 days) all the metabolites were almost entirely utilized, except compound (**8**), which appeared slowly with time and which was present at high relative abundance after a 38-day incubation.

o-Hydroxycinnamic acid (3) degradation. The same consortium was transferred by successive passages to o-hydroxycinnamic acid 0.05%. In this case, the transformation products were o-hydroxyphenylpropionic acid (**10**) and phenlypropionic acid (**11**) (scheme 3). The transformation occurred after a long lag period (fig. 4) and the reaction leading to the C_1 loss from the side chain was absent.

Discussion. The relative rates of degradation are in this order: para > meta > > ortho. The disappearance rates of the three isomers are different, suggesting a positional selectivity concerning the hydroxyl group on the aromatic ring. The comparison between the behavior of para- and meta-hydroxycinnamic acid shows that compound (**8**) appears after a longer incubation time than compound (**5**), suggesting a more difficult dehydroxylative process from the meta than from the para isomer. This observation is in accord with the results obtained for the anaerobic degradation of caffeic acid, where the dehydroxylation reaction occurred mainly at the para position[2]. This positional selectivity seems, however, to be different in nature from that noted previously with caffeic acid. In the present case in fact, the position of the hydroxyl group influences the rate of disappearance of the hydroxycinnamic acid, whereas in caffeic acid metabolism it influenced the nuclear dehydroxylation reaction, by making it regio-selective.

In the degradation of the ortho isomer, the overall reaction is slower and the position of the hydroxyl group affects not only the utilization rate, but also the fate of the parent molecule. In fact, the lack of the C_1 loss from the side chain could be explained supposing a stabilization of the side chain which prevents its demolition. No stable intermediate was detected, but we could suggest the formation, in the reaction medium, of a six-membered lactone structure (**12**), preventing the side chain degradation. γ- and δ-hydroxyacids exist rarely as such: when prepared they spontaneously lose water and form five- and six-membered ring γ- and δ-lactones[3]. No stable lactone intermediate is possible in the case of m- and p-hydroxycinnamic acid.

Acknowledgments. This work was supported by a CNR-ENEA grant.

1 Andreoni, V., Nali, M., Rindone, B., Tollari, S., Treccani, V., and Villa, M., Ann. Microbiol. *34* (1984) 31.
2 Nali, M., Rindone, B., Tollari, S., Andreoni, V., and Treccani, V., Experientia *41* (1985) 1351.
3 Allinger, N.L., Cava, M.P., De Jongh, D.C., Johnson, C.R., Lebel, N.A., and Stevens, C.L., Organic Chemistry, 2nd edn, p. 183. Worth Publishers Inc., New York 1976.

0014-4754/86/11/121254-03$1.50 + 0.20/0