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REDUCTION OF α,β-UNSATURATED KETONES BY PLANT SUSPENSION CULTURES

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Key Word Index—*Medicago sativa*; Papillionaceae; Lucerne; bioconversion; reduction; α,β-unsaturated ketones.

Abstract—α,β-Unsaturated ketones were reduced by various plant cells grown under different cultural conditions. The stereochemistry of the reduction of (−)-carvone by *Medicago sativa* was found to be identical to that obtained with other organisms.

INTRODUCTION

In previous studies, we have shown the reduction of α,β-unsaturated ketones by aerobic bacteria [1], anaerobic bacteria [2], fungi [3, 4] and protozoa [5]. In order to establish if reduction of α,β-unsaturated ketones is widespread among different types of living organisms, we have extended our studies to include plant suspension cultures. Some reductions of α,β-unsaturated ketones by plant cell cultures have been described. Thus besides the reductions of 3-oxo-Δ-4-steroids in which specific enzymes are involved [6], (+)- and (−)-carvone are reduced by cells of *Nicotiana tabacum* [7] and (+)-pulegone by cells of *Mentha* chemotypes [8]. With (−)-carvone and (+)-pulegone the reported yields are about 10%.

We report here results obtained with plant cell suspension cultures particularly those of *Medicago sativa*. The substrates used were substituted cyclohex-2-en-1-ones. The conversion with cyclohex-2-en-1-one was studied along with the stereochemistry of the reaction with (−)-carvone and the influence of the substituent β to the carbonyl group.

RESULTS AND DISCUSSION

The reduction of cyclohex-2-en-1-one (**1**, 240 mg/l) to form small amounts of cyclohexanone (**2**) was brought about by 24-hr-old cell suspension cultures of *Glycine max* (lactose), cultivated on lactose, *G. max* (glucose), cultivated on glucose, *Catharanthus roseus*, *Vinca minor* and *M. sativa* (Table 1). However, no cyclohexanol (**3**) was formed under the experimental conditions employed (Table 1). In *M. sativa* cultures, the proportion of cyclohexanone to cyclohex-2-en-1-one showed no change with time after two days.

A more detailed study of the bioconversion of cyclohex-2-en-1-one by 24-hr-old cultures of *M. sativa* showed that the yields of reaction products did not increase after 24 hr reaction time (Table 2). The yields of cyclohexanone were much greater than in the preliminary experiments (Table 1), reaching almost 50%. In addition, cyclohexanol (**3**), arising from reduction of the carbonyl group of cyclohexanone, was also present.

Table 1. Reduction of cyclohex-2-en-1-one (240 mg/l) by 24-hr-old cell suspension cultures

Strain	Reaction time (days)	% total 1	2	3
G. max lactose	5	92	8	0
G. max glucose	5	95	5	0
V. minor	5	92	8	0
C. roseus	3	92	8	0
M. sativa	2	89	11	0
	3	90	10	0
	4	88	12	0
	5	89	11	0
	6	90	10	0
	7	87	13	0

Table 2. Reduction of cyclohex-2-en-1-one (240 mg/l) by 24-hr-old cultures of *M. sativa*

Reaction time (hr)	Fresh weight (g/l)	% total 1	2	3
6	61	80	20	traces
24	57	56	40	4
48	40	64	33	3
72	42	60	37	3
96	54	57	40	3

The reduction of the ketone occurred both in the growth phase and in the non-dividing phases (Table 3). In addition, the reaction rate/100 ml culture increased with increase in fresh weight.

A kinetic study of the reduction of cyclohex-2-en-1-one (**1**) by *M. sativa* cells (from 200 to 300 g fr. wt/l) showed that after 24 hr, the reduction of not only the C–C double bond, but also of the carbonyl group had gone to

Table 3. Reduction of cyclohex-2-en-1-one (500 mg/l) by cultures of *M. sativa*

Culture time* (hr)	Growth phase	Fresh weight (g/l)	% total		
			1	2	3
24	Lag	46	58	39	3
96	Log	142	26	58	16
192	Stat.	375	13	57	30

*Culture incubated for 72 hr in the presence of the substrate.

Table 4. Kinetics of the reduction of cyclohex-2-en-1-one by washed cells of *M. sativa*

Reaction time (hr)	Fresh weight (g/l)	% total		
		1	2	3
0.33	312	90	6	4
1	266	78	13	9
2	234	74	17	9
3	212	63	23	14
4	298	39	28	33
6	260	18	46	36
8	258	7	40	53
9	282	8	31	61
24	318	traces	traces	100

completion (Table 4). The data in Tables 3 and 4 cannot be compared.

Two enzymes are evidently involved in the reduction of cyclohex-2-en-1-one, an enone reductase and an alcohol dehydrogenase. It is noteworthy that the degree of complete reduction was lower in the first three hours than thereafter. Substrate toxicity and the fact that large amounts of cells are necessary for the reaction to proceed with these concentrations of cyclohex-2-en-1-one (Tables 2 and 3) might explain this result. To explain the apparent decrease in toxicity of cyclohex-2-en-1-one towards cells of *M. sativa*, as the experiment proceeded, the hypothesis is advanced that there is a sub-population of cells in the culture which is resistant to the toxic effects of the substrate. It may be, however, that as the reduction proceeds the substrate reaches sub-toxic levels. In a previous study [5], we showed that both saturated ketone and alcohol are notably less toxic than cyclohex-2-en-1-one towards micro-organisms.

The above experiments demonstrated that *M. sativa* cells, with enough fresh weight of cells, reduce cyclohex-2-en-1-one, giving, according to the reaction time, a mixture of saturated ketone and alcohol, or saturated alcohol only.

In order to determine the stereochemistry of the reaction, we studied the reduction of (−)-carvone (**4**). All the different isomers of the expected reduction products [dihydrocarvone (**5**) and neodihydrocarveol (**6**)] have been described elsewhere (NMR and optical rotation) [3, 9].

The reaction was performed with a cell culture corresponding to a fresh weight of cells of 184 g/l. After three days reaction, a mixture of 21% (−)-carvone (**4**) ($[\alpha]_J^{25}$ = −61°, e.e. 96%), 27% (+)-dihydrocarvone (**5**) and 52% (+)-neodihydrocarveol (**6**) were obtained. The last two compounds were identified by direct comparison of their physical properties (NMR and [α]) with those of authentic samples prepared after [3].

The stereochemistry of the C–C double bond reduction was the same as that observed with various types of micro-organisms, and particularly, bacteria [1, 2] and fungi [3, 4]. The reduction of the carbonyl group yielded an alcohol of *S* configuration, as predicted by Prelog's rule [10]. *N. tabacum* cells [8] give dihydrocarvone (**5**) (1.5%) in addition to neodihydro-carveol (**6**) (10.7%), and dihydrocarveol (**7**) (1.1%).

In previous work on the reduction of α,β-unsaturated ketones by micro-organisms [3], we showed that the substituent of the double bond, β to the carbonyl must be hydrogen.

Similarly, with *M. sativa*, whatever the weight of cells, no reduction product of (**8**) was observed. Thus indicating the need for a H atom in the position β to the carbonyl group.

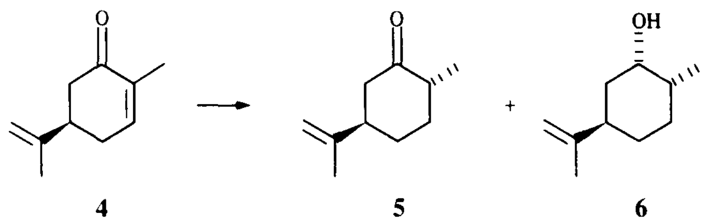
All the results described show that the reduction of α,β-unsaturated ketones is general among biological systems. Tests have shown that the reduction reaction also occurs in rat liver extracts (Fauve, A., personal communication).

EXPERIMENTAL

Materials. Cyclohex-2-en-1-one, 3-methylcyclohex-2-en-1-one and (−)-carvone were commercial products.

Analytical GC: 3 m long stainless steel column filled with 20% Carbowax 20 M on Chromosorb W, H_2 1 Bar, oven temp. 90° (cyclohex-2-en-1-one) or 140° (3-methylcyclohex-2-en-1-one and (−)-carvone). Optical rotations were determined with an automatic polarimeter at 25° for the mercury *J*-line (λ = 578 nm) in $CHCl_3$ soln. ^1H NMR (60 MHz) spectra were recorded in $CDCl_3$ with TMS as int. standard.

The five plant tissue culture strains used for these studies has been established several years previously. The strains were

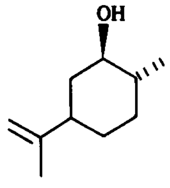


4 **5** **6**

$[\alpha]_D^{20}$ = −62°
Lit. $[\alpha]_D^{20}$ = −62.5° [13]

$[\alpha]_J^{25°}$ = + 16° (*c* 0.03)
Lit. $[\alpha]_D$ = + 14° [14]

$[\alpha]_J^{25°}$ = + 36° (*c* 0.05)
Lit $[\alpha]_D$ = + 33° [15]



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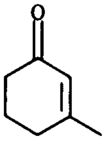

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subcultured every week into conical flasks (250 ml) containing 100 ml of medium and 5 g wt of cells at 26° under 16 hr photoperiod (2500 erg/cm^2), except *Medicago sativa* which was cultivated in darkness, on a gyratory shaker (100 rpm).

Vinca minor L. 13 and *Catharantus roseus* G. Don C20 were cultivated in Gamborg's B5 medium [11] with 2,4D (1 mg/l), kinetin (0.06 mg/l) and sucrose (20 g/l). Two strains of *Glycine max* L. Merr were cultivated on the same medium but without kinetin and with glucose (20 g/l) for the strain named *G. max* glucose and lactose (20 g/l) for the strain named *G. max* lactose. Culture conditions for the *M. sativa* strain have already been published [12].

Extraction procedure. The cell suspension was homogenized using an Ultraturax homogeniser (20 000 rpm for 3 min). The homogenized suspension was satd with $(NH_4)_2SO_4$ and extracted (× 4) with Et_2O. The Et_2O extracts were dried (Na_2SO_4), the solvent evapd off on a water-bath at 45° and the residue analysed by GC. The products were identified by comparison of R_ts with those of authentic samples. In all tables, given yields are relative ones. The total yield is about 75–80% (determined by GC with internal standard).

Reduction of (−)*-carvone.* To 1.6 l of cell suspension of *M. sativa* (3 days old; 184 g/l fr. wt) was added 0.480 g (−)-carvone. The suspension was stirred for 3 days at 26°, then treated as above. The residue was separated and purified by CC on Merck 70–230 mesh silica with pentane–Et_2O (9:1) as eluent to give 21% (−)-carvone, 27% (+)-dihydrocarvone and 52% (+)-neodihydrocarveol.

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