THE ESTIMATION OF UMBELLIFERONE IN HYDROXYLATION OF COUMARIN BY ASCORBIC ACID SYSTEM

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The formation of 7-hydroxycoumarin from coumarin in the non-enzymic (ascorbic) system was demonstrated. Umbelliferone is formed in a low yield, together with a major amount of unidentified material. Separation of the compound was accomplished by TLC. Fluorometric estimation of umbelliferone is described. Statistically designed experiments were used to establish the main effects of some hydroxylating system component.

A comparison of the products obtained by chemical hydroxylation with those yielded by enzymatic hydroxylating systems has shown that although with some compounds both systems furnished the same products, with other compounds there could be marked differences[1]. The enzymic hydroxylation of coumarin in rat and/or rabbit liver microsomes as well as the identification of hydroxylated products in rat urine has been reported[2]. In contrast, relatively little has been done on the non-enzymic hydroxylation of coumarin. It seemed therefore of interest to attempt hydroxylation of coumarin by the ascorbic acid hydroxylating system.

The typical assay procedure for non-enzymic hydroxylation includes the following variables or factors: ascorbic acid, Fe^{2+}, ethylenediaminetetraacetic acid (EDTA),

TABLE I

Values Assigned to the Factors

Coumarin 6·8 μmol, total volume 3 ml, incubation time 2 h, 37°C.

Symbol	Factor	High	Low
A	ascorbic acid, μmol	10	0
B	$FeSO_4$, μmol	0·95	0
C	EDTA, μmol	4·5	0
D	pH (phosphate 0·067M)	7·30	5·50
E	oxygen gas	O_2	N_2

pH, and oxygen gas[3]. Five-factor experiments with two levels of each factor, followed by an analysis of variance, seemed to be well suited for exploratory work on the effects of several factors, and for investigating interactions among those factors[4,5]. The values assigned to the variables tested in this design, are shown in Table I.

TABLE II
Yields of the Different Combinations of Treatments (Relative Fluorescence) in Blocks I—III

Treatment[a]	I	II	III
—	0	0	0
a	7	12·5	10
b	0	0	0
ab	20	15·5	13
c	0·5	0	0
ac	10	3	2·5
bc	0	0·5	1
abc	20·7	31·5	32·5
d	0·5	0	0
ad	2·5	3·5	1
bd	0	0	0·4
abd	7	3·5	4
cd	0	0·5	0
acd	8·5	4·5	5
bcd	4·5	0	0
abcd	37·5	32·5	48
e	0		
ae	30·7	47·2	37·5
be	0		
abe	78·7	79·7	75·7
ce	0·5		
ace	65·5	116·2	25·5
bce	0	0·5	1
abce	48·2	46·5	50
de	0·5	0	0
ade	51·5	35·5	26·7
bde	0	0	0·5
abde	34	37·5	32·5
cde	0	1	0
acde	170	180·5	171
bcde	5	0·3	0
abcde	268	269	223·5

[a] Treatments are indicated by small letters, and the symbol — is used to indicate the absence of all factors.

Additional experiments were carried out to test more closely those variables which appeared significant in the screening design. The concentration ranges were changed and shifted either up or down, depending on which level had produced the highest response in the initial design. The response tested was the extent of 7-hydroxylation, as measured by the fluorescence of the umbelliferone formed during the reaction.

EXPERIMENTAL

Materials

Coumarin, m.p. 68–70°C (Bush), and 7-hydroxycoumarin, m.p. 231–233°C (Fluka), were employed after checking their purity by chromatography and spectrophotometric analysis. L-Ascorbic acid was purchased from Carlo Erba. All other chemicals were of reagent grade.

Incubation Procedure and Assays

Series of solutions were prepared to contain the desired amount of ascorbic acid, EDTA and $Fe(SO_4)_2$ in a total volumen of 0·5 ml. Each solution was pipetted into the test tube. Then 3·5 ml of the buffer solution was added. The pH was adjusted with 0·2M-Na_2HPO_4 followed by 10 micromol of coumarin (in 20 μl of ethanol). The test tube was placed in a water bath at 37°C and the incubation was carried out by bubbling oxygen (or nitrogen) through the solution for two hours. Reagent blanks were prepared in the same manner except that the coumarin solution was replaced by ethanol. The aqueous layer (5 ml) was extracted with ether (4× 5 ml). The combined ether extracts were dried (over Na_2SO_4) and evaporated.

The residue was taken up in ethanol (7 ml) and 10 μl of this solution were chromatographed on thin-layer plate of silica gel G (Merck, Darmstad, German Federal Republic). Test spots containing 0·3 μg of the pure compound were run alongside the unknowns. The chromatogram was developed over 10 cm with chloroform. The intensively blue fluorescent spots showing the same R_F value as umbelliferone under UV light were removed from the plate and transferred to centrifuge tubes. Elution was carried out by adding 1 ml ethanol in the tubes. After centrifugation an aliquot of the supernatant was diluted to a convenient volume with carbonate–bicarbonate buffer, pH 10·2, and the resulting fluorescence was determined by using a Beckman Ratio Fluorometer at an exiting wavelength of 360 nm and measuring the emission through a Kodak Wratten 47 B secondary filter. The eluates of the test spots were used for calibrating the fluorometer to 100% fluorescence. The UV spectra of the eluates and the shifts on addition of sodium acetate and sodium hydroxide were identical with those of umbelliferone. These absorption spectra were measured on a Beckman D.B.G. recording spectrophotometer. When the chromatograms were sprayed with Gibbs reagent, the umbelliferone spots gave negative reactions, but other positive spots were observed.

RESULTS AND DISCUSSION

In the screening experiment (Table II) the following effects were judged significant at the 1% level: ascorbic acid, oxygen gas, Fe^{2+}, EDTA, pH, and all two-factor interactions among them, except the oxygen–Fe interaction. For these data it seemed logical to begin by examining the effects of factors A, B and C from Table I at pH = = 7·30 in the presence of oxygen (Table III). Under the conditions used, incubation

of coumarin resulted in a small but measurable formation of umbelliferone. The yield was estimated to be less than 1%, as measured fluorometrically. In the absence of oxygen (presence of nitrogen), the ascorbic system did not show any, or showed

TABLE III

Analysis of Variance and Examination of Interactions Between Factors A, B and C

Source	D.F.	Sum of squares	Mean square	F
A	1	92 802	92 802	978
B	1	2 273	2 273	23
C	1	47 668	47 668	476
AB	1	2 109	2 109	21
AC	1	46 728	46 728	467
BC	1	2 667	2 667	27
ABC	1	2 408	2 408	25
Treatments	7	(196 738)	28 105	296
Blocks	2	436	218	2·3
Residual	14	1 327	94·8	
Total	23	198 501		

TABLE IV

Effect of Ascorbic Acid on the Umbelliferone Formation

The standard procedure was used but with: EDTE 1·16 μmol, Fe^{2+} 0·127 μmol, coumarin 10 μmol, 0·067M phosphate, pH 7·30 to 5 ml. Incubation time 90 min.

Ascorbic acid, μmol	0	1	3	4	10	20	40	60
Umbelliferone, μmol	11	45	78	118	309	597	1 000	1 400

TABLE V

Effect of Time of Incubation on the Umbelliferone Formation

Coumarin 170 μmol; ascorbic acid 1340 μmol; Fe^{2+} 26 μmol; EDTA 126 μmol; phosphate buffer 0·067M pH 7·30 to 80 ml.

Time, min	5	15	30	60	120	250
Umbelliferone, μmol	0·5	2·0	3·5	6·6	12	22

TABLE VI

Effect of Concentration of EDTA on the Umbelliferone Formation

Coumarin 6·8 μmol; ascorbic acid 10 μmol, Fe^{2+} 1 μmol; phosphate buffer pH 7·30 to 3 ml, incubation time 2 h. Figures given are the average of duplicate determinations.

EDTA, μmol	0·45	0·90	2·25	4·5	22·7	45·5
Umbelliferone, μmol	0·11	0·15	0·19	0·19	0·17	0·20

TABLE VII

Effect of Concentration of Fe^{2+} on the Umbelliferone Formation

Coumarin 6·8 μmol; ascorbic acid 10 μmol; EDTA 4·5 μmol; phosphate buffer 0·067M pH 7·30 to 3 ml. Incubation time 2 h. Figures given are the average of triplicate determinations.

$FeSO_4$, μmol	0·6	1	2
Umbelliferone, μmol	0·15	0·17	0·19

at most only a restricted hydroxylated activity, possibly due to the fact that strict anaerobiosis was not achieved in the system.

A better yield was obtained when both molecular oxygen and ascorbic acid were present during the reaction. Separate experiments, with the other variables hald constant, showed that the amount of umbelliferone formed increased with ascorbic acid (Table IV) and time of incubation (Table V), while no significant effects were observed with different concentrations of EDTA, (Table VI) or Fe^{2+} (Table VII), although the response was higher when the last-named factors were presented in the system (Table III).

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