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Microbiological Transformation of Steroids

XV. Transformation of Steroid S (Reichstein) by *Absidia orchidis* 310

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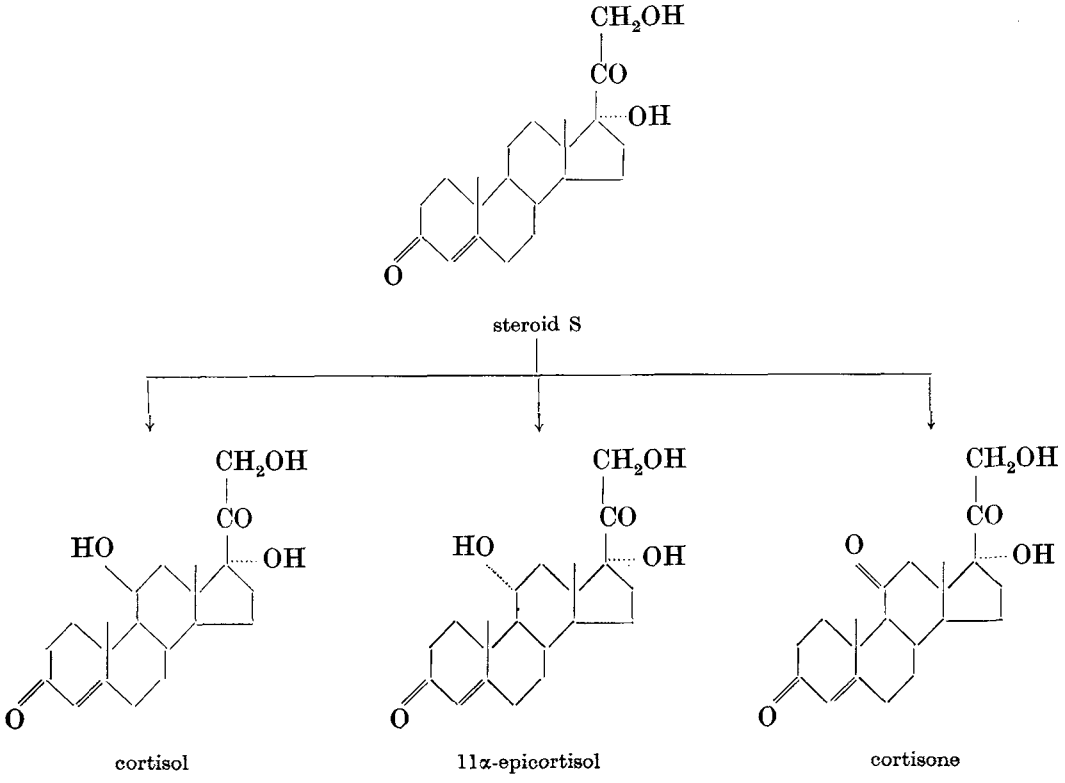
The hydroxylation capacity of 28 different microbial species (a total of 64 strains) was tested in work described previously (Čapek & Hanč, 1961) and it was found that of this number as few as 10 species (19 strains) will hydroxylate steroid S (Reichstein) in the 11α-position. In all the strains of this series steroid S yielded more metabolites, the strain *Absidia orichidis* 310 being apparently relatively the most suitable. The enzymic system of this microorganism will transform steroid S into cortisol and into its 11α-epimer. Another oxidation product, cortisone, is also formed in traces.

The chosen strain of *Absidia orchidis* was then studied further, particularly with respect to the individual factors



affecting the rate of transformation of steroid S to a mixture of cortisol and its 11α-epimer.

Three methods were used for the analysis of the steroid metabolites: (1) spectrophotometry in the u.v. region, using extinction at 242 mμ. Cortisol, its 11α-epimer (epicortisol) as well as the original steroid S, show the same position of the maximum; the extinction values of cortisol and of its epimer are indentical while the extinction of the S compound is relatively higher. (2) Polarography based on the reduction of the conjugated double bond $O{=}\overset{|}{C}{-}\overset{|}{C}{=}C<$ (Eisenbrand & Picher, 1939). There is no difference in the polarographic behaviour of the three compounds. (3) Colorimetry was carried out using the Porter and Silber reaction (Porter & Silber, 1950) with phenylhydrazine in ethanolic solution. The method may only be applied to crude mixtures where both steroids (cortisol and epicortisol) are present in a ratio of 1 : 1, according to a calibration curve constructed for both steroids. The 11α--epimer of cortisol produces about 20% more intense coloration under identical experimental conditions.

MATERIALS AND METHODS

Microorganism. The optimum conditions for the transformation of the steroid S to cortisol and its 11α-epimer were studied on the strain *Absidia orchidis* 310 (obtained from the Department of Fermentation Chemistry, University of Technical Science, Prague).

Cultivation. The strain *Absidia orchidis* 310 was grown under sterile conditions in 100 ml. medium D (white dextrin 3.0%, corn-steep liquor 0.3%—referred to dry weight, $(NH_4)_2HPO_4$ 0.3%, KCl 0.04%, $MgSO_4$ 0.04 %, $FeSO_4$ 0.001%). The pH of the medium was adjusted to 6.2 prior to sterilization, using 10% NaOH. The medium was placed in 750 ml. Erlenmeyer flasks on a reciprocal shaker (70 strokes/min., amplitude 6 cm.) at 27°C for 48 hr.

When the cultivation was terminated (the pH dropped to 4.2—4.5) the contents of 3 flasks were combined, the mycelium filtered off and remainder of the medium removed by washing the mycelium with 150 ml. physiological saline. The washed mycelium was immediately transferred to a flask (1000 ml. capacity) placed on a laboratory stirrer and the volume made up to 300 ml. with a phosphate buffer of known pH.

On account of the fact that the transformation of the steroid on the laboratory stirrer took place under non-sterile conditions, aqueous solution of chlortetracycline (100 μg. per 100 ml. medium) was added to each flask on the stirrer to prevent contamination during transformation.

Transformation of the steroid. The effect of pH, temperature, type and amount of solvents, concentration of added steroid S, number of revolutions of the stirrer and aeration, in the course of transformation of the steroid S was investigated. In studying these factors, a single factor was altered in each series. Every estimation was performed in three mutually independent experiments and the average of these three used for the evaluation.

Analytical methods. The course of transformation was evaluated qualitatively with the aid of paper chromatography (the presence of the original steroid S and the number and types of metabolites produced) and quantitatively by spectrophotometry after elution of the metabolites with ethanol from an undetected paper chromatogram (amounts of cortisol and 11α-epimer).

Whatman paper 4 impregnated with a mixture of formamide-ethanol (1 : 1) was used for chromatography. Chloroform served as the mobile phase. Samples for chromatography were removed in quantities corresponding to 1 mg. original steroid; they were extracted twice with chloroform, the extracts were combined

and evaporated. The residue was dissolved in a small amount of chloroform and the solution applied to chromatographic paper. After developing and drying, steroids were detected on the chromatogram with a mixture of *p*-phenylenediamine and phthalic acid (Bodanszky & Kollonitsch, 1955).

For quantitative spectrophotometry the chromatographic spots of cortisol and of its 11α-epimer were marked on the undetected chromatogram on the basis of extinction of their fluorescence in u.v. light.

The spots were cut out from the paper and cut up into 3 mm.² squares which were eluted for 1 hr. with 10 ml. absolute ethanol, and 0.05 ml. concentrated HCl. The extract was quantitatively transferred to a 25 ml. volumetric flask and ethanol added up to the mark. Extinction was then measured at 242 mμ. An extract from the same area of chromatographic paper without sample was used as blank. A calibration curve was used for quantitative evaluation, the curve being constructed from the extinction values of extracts of chromatograms with standard amounts of the compounds (50, 100, 200 and 500 μg.). Both isomers were extracted from the paper to 95%.

A Heyrovský polarograph ČZ V 301 with a mercury dropping electrode was used for polarographic estimations (t = = 2.6/sec, H = 60 cm., m = 2.95 mg./sec., 4 V battery, Kalousek's flask with a saturated calomel electrode, nitrogen bubbled through the mixture at the beginning); an 0.2 M acetate buffer of pH 4.63 was used (0.2 M acetic acid, 0.2 M sodium acetate 1 : 1). The standard solution of 25 mg. cortisol and of its 11α-epimer was made in 25 ml. rectified 96% ethanol. A calibration curve was constructed for 0.5, 1.0, 1.5 and 2.0 ml. standard solution, ethanol added to 2 ml., then 1 ml. buffer and distilled water up to 10 ml. Nitrogen was bubbled through the solution in the polarographic flask for 5 min. The curves were registered at 1/15 sensitivity. The half-wave potential was about 1.5 V. The height of waves are plotted against concentration in mg./10 ml.

For the estimation, 25 mg. samples of steroid mixture were prepared, dissolved in 25 ml. ethanol and 1 ml. and 2 ml. amounts of solution used for two estimations.

$$\% \text{ steroid} = \frac{mg_n \cdot 25 \cdot 100}{n} \text{ for 1 ml. and}$$

$$\% \text{ steroid} = \frac{mg_n \cdot 12.5 \cdot 100}{n} \text{ for 2 ml.,}$$

where mg_n is the number of mg. read from the calibration curve and n is the number of mg. per 25 ml.

The error of estimation does not exceed 2%. When steroids were estimated directly in the fermentation medium, 5 ml. of the medium (about 3 mg. steroids) were shaken with 5 ml. chloroform three times. The combined extracts were shaken twice with 10 ml. distilled water. The chloroform extract was dried with sodium sulphate and distilled to dryness. The residue was dissolved in 2 ml. ethanol; 1 ml. ethanolic solution was transferred to a 10 ml. volumetric flask, 1 ml. ethanol added, followed by 5 ml. buffer and distilled water up to the mark. Polarography was carried out in the same way as with the calibration curve.

For spectrophotometric estimations of cortisol and its derivatives in u.v. light, 50 mg. steroids was dissolved in 50 ml. absolute ethanol or methanol made up to the mark with the same solvent. The measurement took place in a 1.0 cm.

Table 1. Spectrographic characteristics of steroids

Preparation	Wave-length, mμ	$E^{1\%}_{1\text{ cm.}}$
Cortisol	242	436
Cortisol-21-acetate	242	395*
Cortisol-21-hemisuccinate	242	342
Cortisone-21-acetate	238	392

cuvette against pure solvent at 238, 240, 242, 244 and 246 mμ. The value of $E^{1\%}_{1cm.}$ is equal to E . 1000. The average error of estimation is $\pm 2\%$.

When the estimation was to be carried out directly in the fermentation medium, the sample was shaken as above for the polarographic estimation, then dried and chloroform evaporated; the residue was dissolved in 2 ml. ethanol. From this solution, 0.6 ml. was transferred to a volumetric flask and the volume made up to 100 ml. with ethanol. The measurement is the same as above. It holds that the concentration of steroid in 1 ml. $= \frac{E}{444} \cdot 1.67$, where E is the extinction in a 1 ml. cuvette.

RESULTS AND DISCUSSION

The *Absidia orchidis* 310 strain was cultivated for 48 hr. after which the effect of the individual factors on the course of transformation of the steroid S was investigated.

The effect of pH. The transformation took place under aeration and stirring ($^1/_2$ volume air per min., 200 revolutions per min.). Sixty mg. steroid S in 6 ml. methanol was added to 300 ml. buffer solution. The temperature during transformation was 27°C. The results are expressed in % referred to the weight of steroid S added.

Table 2. The effect of pH on the course of transformation of steroid S

pH	Incubation in hr.					
	15		20		24	
	Cortisol %	Epicortisol %	Cortisol %	Epicortisol %	Cortisol %	Epicortisol %
4.4	18.6	17.2	20.0	19.8	23.8	22.4
5.5	44.1	42.8	46.2	44.6	46.0	44.3
6.5	49.7	44.1	48.9	43.9	48.9	43.6
7.3	43.8	40.9	44.6	42.8	45.8	43.0
8.0	27.1	25.4	30.0	26.1	33.5	28.2

The pH optimum for the transformation of the steroid S by *Absidia orchidis* is in the vicinity of 6.5. At this pH the maximum of transformation was reached during the 15th fermentation hour. A partial decrease or increase of the pH value (to 5.5 or 7.3) will retard the course of transformation. At 4.4 or 8.0 considerably lower yields of cortisol and of its 11α-epimer were obtained under the same conditions.

When other effects on the course of transformation were studied the pH of the solution was always set at 6.5 before adding the steroid.

The effect of temperature. The transformation took place under aeration and stirring ($^1/_2$ volume air/min. and 200 r.p.m.). Sixty mg. steroid S in 6 ml. methanol was added to 300 ml. buffer solution (pH 6.5).

The effect of temperature on the course of transformation was evaluated during the 18th hour. The results are expressed as a percentage with respect to the weight of added steroid S.

Table 3. The effect of temperature on the result of 18-hour fermentation of steroid S

Temperature °C	Cortisol %	Epicortisol %
24	39.8	37.2
27	46.8	45.1
30	45.2	45.1
33	38.8	37.9

The temperature optimum lies in the range of 27—30°C. Lower and higher temperatures retard the course of transformation.

The effect of the type and quantity of solvent. The transformation took place under aeration and stirring ($^1/_2$ volume air per min. and 200 r.p.m.). Sixty mg. steroid S was added to 300 ml. buffer solution (pH 6.5), after dissolving in 6, 12, 18 and 24 ml. given solvent. Methanol, ethanol and acetone were used. The

temperature during transformation was 27°C. The evaluation of the effect of solvents was made during the 18th fermentation hour. The results are expressed as a percentage with respect to the weight of steroid S added.

Table 4. The effect of the type of solvent on the result of 18-hour fermentation of steroid S

Solvent ml.	Methanol		Ethanol		Acetone	
	Cortisol %	Epicortisol %	Cortisol %	Epicortisol %	Cortisol %	Epicortisol %
6	47.8	43.5	44.5	39.8	46.1	40.0
12	46.5	42.6	38.2	30.1	39.1	32.1
18	40.0	36.2	22.8	20.09	24.1	20.2
24	21.0	24.2	4.1	11.3	—	14.0

Methanol proved to be the most suitable of the solvents tested when used in concentrations of 2—4 ml. per 100 ml. medium. At higher concentrations (6—8 ml. per 100 ml. medium) it markedly blocks the course of transformation. Ethanol and acetone proved to be less suitable (slower transformation) in the given concentrations than methanol.

The effect of number of revolutions. Transformation was tested under aeration ($^1/_2$ volume air per min.) and stirring (200, 400—600 r.p.m.). Sixty mg. steroid S in 6 ml., methanol was added per 300 ml. buffer solution (6.5). The temperature used was 27°C. The experiment was evaluated during the 12th hour of fermentation. The results are expressed as a percentage with respect to the weight of steroid S added.

All the preceding experiments (the effect of pH, temperature and type of solvent) were carried out at a constant number of r.p.m. (200). The maximum transformation was attained during the 15th-18th hour of fermentation. On increasing the number of r.p.m. to 400—600 the fermentation period required for the transformation of steroid S is shortened to 12 hours but on account of conditions in the production and existing technological equipment it was not possible to apply increased numbers of r.p.m. for diminishing the transformation period of steroid S. Therefore, all further experiments were carried out at 200 r.p.m.

The effect of aeration. The transformation was carried out under stirring (100 r.p.m.). Sixty mg. steroid S in 6 ml. methanol was added to 300 ml. buffer solution (pH 6.5). The temperature during transformation was 27°C. The fermentation medium was aerated with $^1/_2$ air volume per min. Transformation without aeration was studied simultaneously. The results are expressed as a percentage with respect to the weight of steroid S added, during the 18th hour of fermentation. It follows from the results that aeration with $^1/_2$ air volume is sufficient for satisfactory transformation. By increasing the volume of passing air the rate of transformation is not affected; when no aeration of the substrate is taking place, however, the rate of transformation is decreased sharply.

The effect of concentration of steroid S. Transformation took place under aeration

Table 5. The effect of the number of revolutions per minute on the result of a 12-hour fermentation of the steroid S

R.p.m.	Cortisol %	Epicortisol %
200	38.4	35.9
400	42.6	40.1
600	46.8	44.6

Table 6. The effect of aeration on the result of a 18-hour fermentation of the steroid S

Amount of passing air	Cortisol %	Epicortisol %
0	12.8	10.3
$^1/_2$ volume	46.8	43.4
1 volume	48.3	42.9

and stirring ($^1/_2$ air volume per min., 200 r.p.m.). Temperature during transformation was 27°. Sixty, 120 and 180 mg. steroid S in 12 ml. methanol was added to 300 ml. buffer solution (pH 6.5). The results are expressed in percentages with respect to the weight of steroid S added.

It follows from the experiment that practically all concentrations of steroid S

Table 7. The effect of the concentration of steroid S on the result of fermentation

Concentration of steroid S in 100 ml. medium	Duration of fermentation			
	18 hours		24 hours	
	Cortisol %	Epicortisol %	Cortisol %	Epicortisol %
20 mg.	46.8	45.1	45.6	44.8
40 mg.	47.1	45.6	47.3	44.8
60 mg.	45.6	44.8	46.9	43.8

are applicable for the given type of transformation. With 60 mg. steroid S/100 ml. medium not only was the transformation period increased but some steroid was left in the medium after fermentation. Therefore, the maximum applicable concentration of steroid S under the given conditions was taken to be 60 mg./100 ml. medium.

SUMMARY

The transformation of steroid S (Reichstein) by the strain *Absidia orchidis* 310 is described, the product containing cortisol and its 11α-epimer epicortisol, and cortisone. A procedure for the analytical evaluation of the transformation of the steroid is given together with an investigation of the effect of pH of the medium (optimum 6.5), of temperature (27—30°C), of type and quantity of solvent (the best is 2—4 ml. methanol/100 ml. medium), of number of r.p.m. of stirring during fermentation (optimum 600 r.p.m.), of aeration and of concentration of the original steroid, on the rate of transformation (optimum up to 60 mg./100 ml. medium). The yield of steroid S transformation under the optimal conditions amounts to 48.9% cortisol and 43.9% 11α-epicortisol, cortisone being formed in traces only.

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МИКРОБИОЛОГИЧЕСКИЕ ПРЕВРАЩЕНИЯ СТЕРОИДОВ.

XV. Изучение трансформации стероида S (Reichstein) штаммом Absidia orchidis 310

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Описана трансформация стероида S (Reichstein) штаммом Absidia orchidis 310 в смеси кортизона, 11-α-эпикортизола и кортизола. Приводится метод аналитической оценки превращения стероида. Было исследовано влияние величины pH среды (оптимум 6,5), температуры (27-30°C), вида и количества растворителя (наиболее удобен метиловый спирт в количестве 2—4 мл. (100 мл среды), числа оборотов при перемешивании в течение ферментации (оптимально 600 об./мин.), далее, влияние аэрации и концентрации исходного стероида на скорость превращения (оптимально до 60мг/100мл среды). При оптимальных условиях выход стероида S, по аналитическим данным, составляет 48,9% кортизола и 43,9 % 11α-эпикортизола; кортизон образуется только в микроскопических количествах.