COMPOSITION OF MEDIA FOR CULTIVATION OF MYCOBACTERIA SPLITTING THE SIDE CHAIN OF SITOSTEROL

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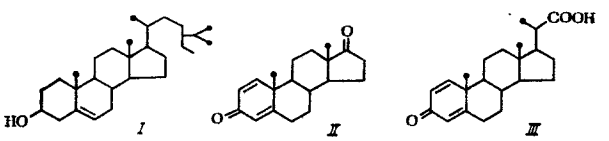
Sitosterol (I) is a promising starting material for the synthesis of medicinal preparations which are steroid hormones, under the condition that a strain of microorganisms is selected which oxidizes the side chain of the molecule to form products suitable for further synthesis.

We have already shown that I can be oxidized by cultures of mycobacteria in the presence of a degradation inhibitor of the steroid skeleton, 8-hydroxyquinoline [1]. In the present work, we studied cultures of *Myc. mucosum* 3-AP and *Myc. flavum* B_2 that in the presence of 8-hydroxyquinoline form oxidation products: androsta-1,4-diene-3,17-dione (II) (the two cultures) and 23,24-bisnorchola-1,4-dien-3-on-22-oic acid (III) (*Myc. flavum* B_2).



I II III

The cultivation of the bacteria and the transformation of I were carried out in media containing a meat–peptone broth and wort (*Myc. mucosum* AP) or a yeast autolysate and casein hydrolyzate (*Myc. flavum* B_2) [1]. The oxidation of I on these media gives variable results because of the instability of the media.

The aim of the present work was to select media with a controllable composition that ensures that reproducible results can be obtained in the oxidation of the side chain of I by cultures of mycobacteria, and to study the regularities of this process.

Transformation Conditions and Oxidation Activity of I

Myc. mucosum 3-AP. We proposed a medium A with the following composition: glucose 0.5%, asparagine 0.04%, ammonium succinate 0.3%, dipotassium hydrogen phosphate 0.03%, magnesium sulfate 0.3%, pH 7.4. The medium ensures the accumulation of a considerable amount of cell mass – 2.2 mg/ml after 24 h of cultivation. It was found that the culture grown on this medium cannot transform I. To verify the assumption that metabolism products that inhibit enzymes able to oxidize I are present in the medium, the transformation was carried out with cells washed free from the medium in a phosphate buffer at pH 7.2. In this case, II and III could be detected in small amounts; hence the assumed products do accumulate in the medium.

As a base for preparing the medium, we used the Peterson medium [2] with the simplest composition, containing the following components: ammonium nitrate 0.15%, dipotassium hydrogen phosphate 0.025%, iron sulfate 0.001%, magnesium sulfate 0.025%, sodium chloride 0.005%, yeast autolysate 10 mg/liter, a colloidal solution of I 20 μg/ml. After 24 h of cultivation, 0.4 mg/ml of cell mass, with low ability to oxidize I, is formed on this medium. When thiamine, biotin, or asparagine in a concentration of 0.24-0.4 mg% was added to this medium, the amount of cell mass increased, but its activity remained low. Addition of a maize extract, meat–peptone broth, wort, peanut or soya oil in a concentration of 50-200 mg% appreciably stimulates both the formation of the cell mass and its oxidizing capacity. The best results were obtained when 2% wort or peanut oil were used. The addition to the main mass of carbon

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sources in a concentration of 1% appreciably influences the amount of cell mass and its oxidizing capacity. The amount of cells after 24 h of cultivation varies from 0.45 mg/ml when glucose is used, to 1.2 mg/ml in the cultivation on mannitol. Mannitol and glycerol completely inhibit, while glucose, saccharose, maltose, lactose, and I at concentration of 20 μg/ml stimulate, the oxidizing capacity of *Myc. mucosum* 3-AP. The oxidation of a 0.5 g/liter solution of I was carried out on a modified Peterson medium, with addition of 1% glucose and 2% of wort, to form 35% of III and 14% of II.

It was found that the transforming activity of the *Myc. mucosum* 3-AP culture is independent of the amount of cell mass, and is strongly dependent on the composition of the culture medium.

Compound I can be transformed with cells washed free from the culture medium in a phosphate, phosphate–citrate, and tris-HCl buffer in a pH range from 5.5 to 8.0. The ratio of the transformation products can be regulated by the amount of the cell mass effecting the transformation of sterol. With a mass of 3.8 mg/ml the maximal amount of III is formed, and with 8 mg/ml, the maximal amount of II.

Myc. flavum B_2. In the cultivation of the culture on the synthetic medium A, after 24 h 0.7 mg/ml of cell mass is formed. When this medium is used, 44% of II is formed; the same yield was obtained when a rich medium was used [1]. Exclusion of glucose from the composition of the medium does not influence either the amount of the cell mass grown, or the amount of II formed. When different carbon sources were compared, it was found that 0.5% glycerine and mannitol inhibit the oxidizing capacity of the culture by 50%; 0.5% saccharose, maltose, and lactose are equivalent to glucose. When 20 μg/ml of I are introduced, basides II, 50% of III is formed, which under different conditions is not formed. When a Peterson medium is used with 0.5% glucose, the amount of cell mass and its oxidizing capacity decrease by a factor of almost 2. Hence, the amine nitrogen is more favorable than the mineral nitrogen. The culture obtains the microelements required from the oxidation from tap water on which the medium is prepared. Exchange for distilled water leads to a 50% decrease in the amount of II formed.

A suspension of *Myc. flavum* B_2 cells actively effects the transformation process of I in the pH range from 6.3 to 8.0.

Thus, the *Myc. flavum* B_2 culture is less sensitive to the cultivation conditions and display of oxidizing capacity than *Myc. mucosum* 3-AP.

Agarized Medium for Sustaining Mycobacteria

Myc. mucosum 3-AP dissociates into S- and R-forms, depending on the composition of the culture medium: The less active R-form grows preferentially on the agarized synthetic medium A, while the active S-form [3] that oxidizes I in the presence of an inhibitor with an appreciably higher yield of the products grows preferentially on the rich agarized medium [1]. The composition of the culture medium also influences the degree of oxidation: the R-form of colonies with agarized medium A oxidizes I to III without an admixture of II.

Myc. flavum B_2 grows on a rich agarized medium and on medium A in the R-form, and therefore its activity towards sterol is independent of composition of the medium.

The above data show that the ability to oxidize I in the presence of a degradation inhibitor with the formation of products suitable for further synthesis is a property that varies with the type of mycobacteria, and in each case this property is most evident under specific conditions for both the cultivation of bacteria and carrying out the transformation of I.

EXPERIMENTAL

The cultures were obtained from the collection of the All-Union Scientific-Research Institute of Pharmaceutical Chemistry. The composition of the media used previously and the cultivation conditions are described in [1]. Compound I was introduced into a 24-h culture in an amount of 0.5 g/liter in the form of a solution in dimethylformamide simultaneously with 8-hydroxyquinoline at a concentration of 15 mg% for *Myc. mucosum* 3-AP and 5 mg% for *Myc. flavum* B_2. The oxidation products on the culture medium after 3 days of transformation was determined by TLC [1].

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OPTIMIZATION OF CYTOCHROME C GRANULATION

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The Leningrad Pharmaceutical Chemistry Institute has developed and perfected the tableting of medicinal form of cytochrome c, tablets containing 0.01 g with an eterosoluble coating [5]. As additives (calculated per 0.01 g cytochrome c), 0.0200 g of sodium chloride, 0.3170 g of sucrose, 0.0030 g of calcium stearate (composition A) or 0.0200 g of sodium chloride, 0.3000 g of lactose, 0.0165 g of starch, 0.0005 g of methylcellulose, and 0.0030 g of calcium stearate (composition B) are used.

During storage of sucrose-containing prototype tablets prepared by the moist granulation method, the cytochrome c content in the tablets decreased by 20% after 12 months due to the effects of residual moisture.

When cytochrome c is granulated in a fluidized-bed apparatus instead of moist granulation, the stability of the tablets during storage increased significantly.

The aim of the present study was to optimize parameters of the granulation process in a fluidized-bed apparatus, thus ensuring cytochrome c tablets of high quality.

Keeping in mind that many factors (quantity, concentration and volume flow of the wetting agent, temperature, conditions of pseudofluidized layer formation, etc.) may influence granule quality, we used the mathematical experiment planning method of Bochs–Wilson [1] for choice of the optimal process parameters.

Experimental studies were carried out in a Strea-1 laboratory apparatus from the Aeromatic company (Switzerland). A single batch of material comprised 0.350 g. Water was used as the wetting agent and sucrose (composition A) as the filler. The spray nozzle was located 0.230 m above the air distributor.

In preliminary experiments five factors were found to exert the most influence on the granulation process (Table 1). The relative error of the results of two parallel experiments did not exceed ±5%, indicating that the experiment may be considered reproducible.

Friability was chosen as the parameter of optimization and was estimated according to the mass discharge rate [3, 8]. The matrix and the experimental results are presented in Table 1.

The results of statistical analysis [6] are as follows: Cochran values, $G_{exp} = 0.36$ and $G_{calc} = 0.68$; variance of the optimization parameter, $S^2(Y) = 0.78$; variance of the deviation of coefficient determination, $S^2(b_i) = 0.049$; rms deviation of the variance of regression coefficient, $S(b_i) = 0.22$: Student's t value = 2.3; variance of model adequacy, $S_{ad}^2 = 2.87$; Fisher values, $F_{exp} = 3.65$ and $F_{calc} = 4.07$.

From statistical analysis of the experimental results presented in Table 1, it follows that the coefficients b_2, b_3, and b_4 were insignificant (b_2, b_3, $b_4 < t = 0.05 \times S(b_i) = 0.51$). When they are excluded, the regression equation become

$$\hat{Y} = 83.4 + 1.19\, x_1 + 2.68\, x_5.$$

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