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Biotransformation of Steroids by a Recombinant Yeast Strain Expressing Bovine Cytochrome P-45017α

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Abstract—The cDNA encoding cytochrome P-45017α from bovine adrenal cortex was expressed in *Saccharomyces cerevisiae* under the control of the galactose-inducible *GAL10* promoter. Carbon monoxide difference spectra of the galactose-induced yeast cells showed expression of about 240 nmol of P-45017α per liter of the culture. Binding of progesterone to the cytochrome P-45017α was clearly detectable already with intact yeast cells as judged by the formation of type I substrate difference spectra. Yeast cells grown on minimal medium containing galactose actively converted progesterone to 17α-hydroxyprogesterone, this indicating the functional integrity of the heterologously expressed P-45017α and its efficient coupling with the constitutive NADPH-cytochrome P-450 reductase. More than 80% of the metabolite produced was secreted into the culture medium. Cultivation in a rich non-selective medium resulted in the formation of an additional product, which was identified by mass spectrometry as 17α-hydroxy-20-dihydroprogesterone. Kinetic analysis revealed that its production followed the cytochrome P-45017α-dependent hydroxylation reaction. The reduction of the 20-keto group of 17α-hydroxyprogesterone was also observed in the non-induced yeast culture, this suggesting the involvement of the constitutive enzyme. Among several substrates tested, progesterone was hydroxylated by the cytochrome P-45017α expressed with the highest activity. The activity towards other substrates decreased in the sequence: 11β- > 11α- > 19-hydroxyprogesterone. In conclusion, the present results show that the host–vector system used is suitable for high-level functional expression of P-45017α and further application of enzymatic properties of this protein to perform specific steroid biotransformations.

Key words: cytochrome P-45017α, recombinant yeast *S. cerevisiae*, 17α-hydroxyprogesterone, 17α-hydroxy-20-dihydroprogesterone, 20-ketosteroid reductase

Virtually all monooxygenase systems from mammalian adrenal cortex exhibit several alternative activities that are catalyzed by a single protomeric protein form: cytochrome P-450scc (two hydroxylation reactions and cleaving of the C_{20}–C_{22} bond), cytochrome P-45011β (three reactions catalyzing hydroxylation of a steroid molecule at the 11, 19, and 18 positions with different specific activities), cytochrome P-45017α (hydroxylation at the C_{17} position and the lyase reaction at the C_{17}–C_{20}). Thus, a dynamic model is realized that includes substrate sites of different P-450 cytochromes determining a site- and stereo-specificity of the reactions and the mobile heme-containing oxidant. This system gives rise to the multiple activities [1] and, consequently, suggests the possibility of shunting of pathways of steroid biosynthesis in cases of different molecular pathologies connected with mutations of the enzymes involved in steroidogenesis. Compartmentalization of enzymes involved in steroid biosynthesis in different tissues (adrenal cortex, testis, placenta) determines the strict consequence of pathways of synthesis of corticosteroids, progestins, and sex hormones, and quantitative differences in the content of the enzymes, mainly in the concentration ratio of cytochrome P-45017α and cytochrome b_5 in different steroidogenic tissues, direct biosynthesis into the corticosteroid pathway or into the pathway of androgens and estrogens [2-4].

Abbreviations: P-45017α) cytochrome P-450 from microsomes of bovine adrenal cortex encoded by the *CYP17* gene and catalyzing the reaction of 17α-hydroxylation of progesterone and pregnenolone.

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Considering the phylogenetic conservativeness of the molecular structure of monooxygenase systems, the use of transgenic microorganisms bearing genes of mammalian cytochromes P-450 is a promising model for investigation of various pathways of steroid biotransformation. While studying the role of cytochrome P-45017α in regulation of steroidogenesis [5, 6] and in coupling of the reactions catalyzed a the foreign heme protein and endogenous electron-transport proteins of microorganisms [7-12], and also considering potentiality of application of transgenic organisms containing different cytochromes P-450 for biotechnological synthesis of steroids [13-15], special attention should be given to the following problems: 1) the possibility of the formation of side products; 2) relation between the systems of steroid transport to foreign proteins and steroid biotransformation; 3) excretion of the modified steroids out of cells as potential xenobiotics for the microorganisms.

In the present work we used a transgenic strain of *Saccharomyces cerevisiae* with an inserted gene of cytochrome P-45017α from bovine adrenal cortex to study the pathways of steroid transformation and to identify possible side products.

MATERIALS AND METHODS

In this work we used the following chemicals: progesterone, 17α-hydroxyprogesterone, 11α-hydroxyprogesterone, 11β-hydroxyprogesterone, 19-hydroxyprogesterone, deoxycorticosterone, dexamethasone, androstendione, Tween 20, EDTA, and dithiothreitol from Sigma (USA); deoxycortisol, testosterone, and metirapon from Serva (Germany); acetonitrile from Fluka (Switzerland); sodium cholate from Calbiochem (USA).

Microorganism strains. The original strain was *Saccharomyces cerevisiae* GRF18 (*Matα his3-11 his3-15 leu2-3 leu2-112 cir*$^{+}$ *can*^{R}) [16]; transformants were *S. cerevisiae* YEp51 (negative control strain) and *S. cerevisiae* YEp5117α (inserted gene of cytochrome P-45017α).

The pCMV plasmid containing a full-length (1800 bp) cDNA encoding cytochrome P-45017α from bovine adrenal cortex [17, 18] was kindly provided by Prof. Waterman (Vanderbilt University, Nashville, USA). To construct the expression vector YEp5117α (Fig. 1), the pCMV plasmid was cleaved by *Hind*III and *Eco*RI, yielding two fragments of cDNA of cytochrome P-45017α: *Hind*III-*Eco*RI (280 bp) and *Eco*RI-*Hind*III (1520 bp), which were cloned into the pUCBM21 vector (2725 bp). The larger fragment encoding the C-terminus of cytochrome P-45017α was ligated without additional modifications into the vector plasmid cleaved by the *Eco*RI/*Hind*III restrictase, yielding the pCF plasmid (4150 bp). To modify the 5′-non-coding sequence, the following oligonucleotide linkers were used (the ATG start of the translation is shown bold):

oligonucleotide 17A1 5′-TCGACA**ATG**TGGCTGCTCCTGGCTGTC-3′
oligonucleotide 17A2 3′-GT**TAC**ACCGACGAGGACCGACA-5′

The smaller fragment *Hind*III-*Eco*RI encoding the N-terminus of cytochrome P-45017α was cleaved by *Hph*1, yielding a fragment of 253 bp. After addition of the 17A1 and 17A2 oligonucleotides, the fragment was ligated with the pUCBM21 vector cleaved by *Sal*I/*Eco*RI to yield the pNF plasmid (2950 bp). The *Sal*I-*Eco*RI fragment of the pNF vector and the *Eco*RI-*Hind*III fragment of the pCF vector were ligated into the high copy-number yeast shuttle vector YEp51 (7672 bp, *LEU2*, 2μ ARS) cleaved by *Sal*I/*Hind*III [19]. The resulting vector YEp5117α (8710 bp) provides a high level of expression of cDNA of cytochrome P-45017α from bovine adrenal cortex under the control of the *GAL10* promoter in *S. cerevisiae*, as described earlier for other genes of cytochromes P-450 [20].

Cultivation of transformants. The yeast was grown in minimal synthetic YNB medium: 1.34% yeast nitrogen bases without amino acids (composed according to Difco (USA) with slight modifications), 100 μg/ml L-His, and 2% glucose [21, 22] or in YPD (Difco): 1% yeast extract, 2% peptone, and 2% glucose [23]. In both cases the yeast was cultivated at 30°C and 180 rpm for 24 h. Cytochrome

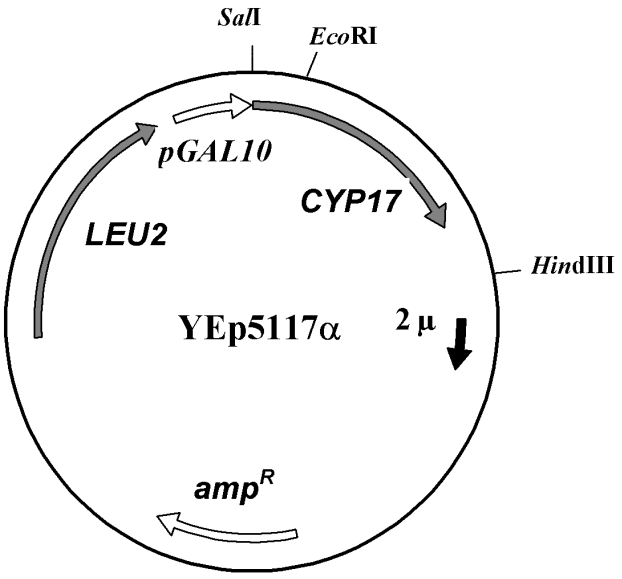


Fig. 1. Scheme of the autonomously replicating high copy-number (50-100) YEp5117α plasmid (8.71 kb) providing functional expression of bovine cytochrome P-45017α in *S. cerevisiae* under the control of the *GAL10* promoter. The *CYP17* cDNA encoding cytochrome P-45017α from bovine adrenal cortex was cloned in several steps from the pCMV plasmid [18] into the yeast–*E. coli* YEp51 shuttle vector containing the homologous *LEU2* gene (yeast selectable marker), *amp*^{R} (*E. coli* selectable marker), and a part of 2μ DNA as the ARS in yeast (see "Materials and Methods").

P-45017α synthesis was induced by addition of D-galactose (2%) after complete consumption of glucose, and then the yeast was grown for an additional 24 h.

Biotransformation of progesterone during the induction and expression of cytochrome P-45017α. Progesterone was added to the cell cultures simultaneously with the induction of cytochrome P-45017α and at 24 h after the induction. The steroid was added as an ethanol solution (10^{-2} M) to the final concentration of 100 μM. The cultures were incubated in 200 ml shaken flasks at 28°C and 180 rpm. After different time intervals, samples (2 ml) were taken from the incubation mixture and centrifuged at 8000 rpm to remove the cells. The supernatant was extracted twice with 4 ml of ethyl acetate, and the pooled organic phase was evaporated using a rotary evaporator. The dry residue was dissolved in 200 μl of methanol.

Isolation and purification of products of biotransformation of progesterone and its monohydroxylated derivatives. Preparative thin layer chromatography (TLC) was performed on UV-254 silica gel plates (10 × 5 cm, Merck, Germany) using benzene–acetone (4 : 1 v/v) as the solvent system. Samples were applied on the plate together with steroid standards (progesterone, androstendione, 17α-hydroxyprogesterone, deoxycorticosterone, and deoxycortisol). After the separation, bands corresponding to different products were scraped off, ground, and extracted twice with 4 ml volumes of methanol. The pooled extract was evaporated; the dry residue was dissolved in 100 μl of methanol and purified using HPLC.

High performance liquid chromatography (HPLC) was performed on an LC-10AT chromatography system (Shimadzu, Japan) using an SPD-M10A diode array UV-detector. To set parameters of the process (gradient shape, elution rate) and to calculate peak areas, the CLASS-VP software (Shimadzu) was used. To analyze steroids, a 5-μm Nucleosil 100-5 C_{18} column (124 × 4 mm, Machery-Nagel, Germany) was used. The chromatography was performed under isocratic conditions using the solvent system acetonitrile–water (60 : 40 v/v).

Mass spectral analysis of isolated transformation products was performed using a QP-5000 chromato-mass spectrometer (Shimadzu). Steroid samples purified by TLC and HPLC were introduced directly into the MS detector (electron ionization, 70 eV). The NIST mass spectral library was used to analyze the data.

Difference absorption spectra of native cells, isolation of microsomes, and analysis of solubilizates by HPLC. At 24 h after the addition of D-galactose, the cells grown in YNB medium were precipitated by centrifugation and washed twice with 0.05 M sodium phosphate buffer, pH 7.2. To determine the content of cytochrome P-45017α, the cells were resuspended in the same buffer ($8 \cdot 10^8$ cells per ml), and then sodium dithionite was added. The mixture was poured into two spectrophotometric cuvettes, and carbon monoxide was bubbled through the experimental cuvette. The difference absorption spectra were recorded for 20 min using an adapter for studying turbid samples (Shimadzu UV 300 spectrophotometer, Japan). The molar absorption coefficient $\varepsilon_{450\text{-}490\ nm}$ was taken as 91,000 [24]. To obtain progesterone-inducible difference spectra, the same concentration of cells was used, but 100 μM progesterone was added into the experimental cuvette, and the maximal spectral response was recorded in 5-10 min. To break the cells, they were ground with glass balls (400-600 μ) for 5 min [25], and a microsomal fraction was isolated using differential centrifugation. Membranes were solubilized for 1 h in the presence of sodium cholate and Tween 20 (final concentrations 0.5 and 0.25%, respectively). The solubilizate was centrifuged at 150,000*g* for 1 h, and the supernatant was analyzed by HPLC using a TSK Phenyl 5PW column (75 × 7.5 mm, LKB, Sweden). Buffer solutions were the following: A) 0.05 M potassium phosphate buffer, pH 7.2, containing 15% glycerol, ammonium sulfate (10% saturation), 0.05% Tween 20, 0.05% sodium cholate, 1 mM EDTA, and 0.1 mM dithiothreitol; B) the same components but sodium cholate concentration was 0.5% and no ammonium sulfate. The conditions for chromatography were: buffer A for the first 5 min, then an exponential gradient of buffer B (5-30 min). Products were detected at 418 nm. Sample volume was 100 μl, elution rate 1 ml/min.

RESULTS AND DISCUSSION

Expression level and functional activity of the cytochrome P-45017α. In a standard experiment on the inducible synthesis of cytochrome P-45017α in the recombinant yeast, both the carbonyl difference spectra and the progesterone-dependent type I difference spectra can be recorded using native cells. It was shown that the total content of cytochrome P-450 determined from the CO difference spectra ($\varepsilon_{450\text{-}490\ nm}$ = 91,000) corresponded to the content of the expressed cytochrome P-45017α determined from the progesterone-inducible type I difference spectra (enzyme–substrate complex formation, $\varepsilon_{390\text{-}420\ nm}$ = 110,000) (Fig. 2). The level of the cytochrome P-45017α expression induced by addition of D-galactose to the culture growing in minimal synthetic YNB medium reached 200-240 nmol per liter culture medium, and the content of the heme protein in the membranes of endoplasmic reticulum was 370-400 pmol per mg total protein. The YEp5117α vector resulted in functional expression of cytochrome P-45017α. Analytical experiments on the biotransformation of [^{3}H]progesterone (10 nmol of the steroid, $5 \cdot 10^7$ cells per ml) with subsequent separation of the products using TLC and radio scanning of the chromatograms revealed a catalytic activity of the transgenic microorganisms: progesterone was transformed mainly to 17α-hydroxyprogesterone with the turnover number of

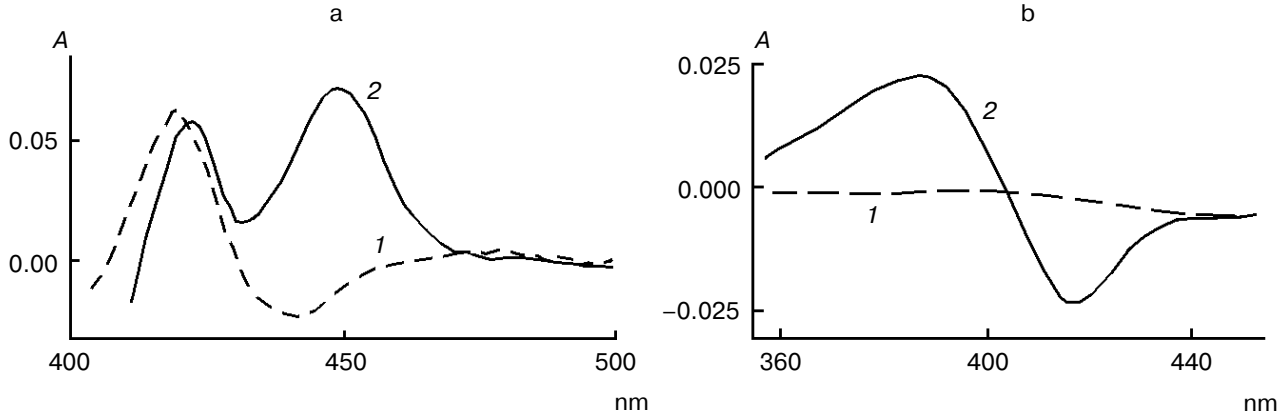


Fig. 2. Expression of cytochrome P-45017α in *S. cerevisiae* detected by CO (a) and progesterone-inducible (b) difference spectra: *1*) control strain of *S. cerevisiae* YEp51; *2*) *S. cerevisiae* YEp5117α.

19 min^{-1} for intact cells and 7 min^{-1} for the membranes of endoplasmic reticulum [21]. HPLC analysis of the solubilizate of endoplasmic reticulum membranes revealed a main peak of the heme-containing protein with retention time of 27.2 min. We also observed peaks with retention times of 2.5 min (5%) and 3.5 min (7%). It should be noted that the yield of the cytochrome P-45017α on purification using HPLC constituted 40% of the expected yield. This can be accounted for by inactivation of the heme protein, its irreversible sorption, and incomplete separation of cytochromes P-450 on the support during the chromatography. Highly purified cytochrome P-450scc from mitochondria of bovine adrenal cortex [26, 27] under the same conditions of chromatography exhibited the retention time of 17.2 min. These differences may be explained by a more hydrophobic nature of cytochrome P-45017α.

Side product of progesterone transformation by the transgenic strain *S. cerevisiae* YEp5117α. It was shown that the change of the selective medium (YNB + $FeCl_3$ + L-His) to the medium for more intensive growth (YPD) results in 2-3-fold increase of cell growth, while the level of D-galactose-induced expression of cytochrome P-45017α per ml of medium remains constant. Figure 3 presents chromatograms of the products of progesterone transformation by the transgenic and control yeast strains while being cultivated on YNB and YPD media. The control strain (*S. cerevisiae* YEp51) growing on both YNB and YPD media did not exhibit any activity (neither hydroxylation nor reduction) towards progesterone: the chromatograms did not reveal any other steroids during 24 h of incubation (Fig. 3, curves *1* and *4*). Chromatograms of the extracts obtained after the incubation of progesterone with *S. cerevisiae* YEp5117α compared to those obtained with the control strain revealed two products of progesterone transformation (Fig. 3, curves *2-6*). The retention time for the product I corresponded to 17α-hydroxyprogesterone

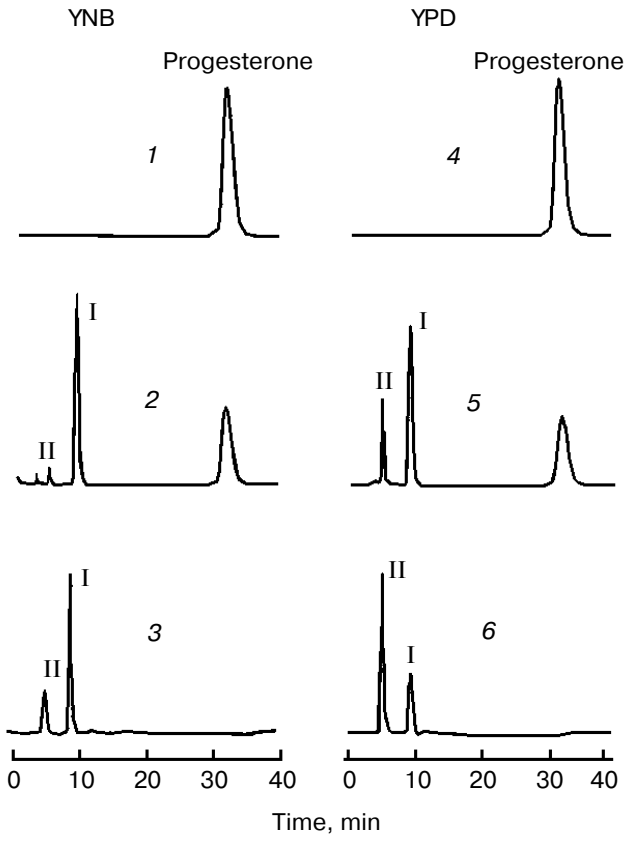
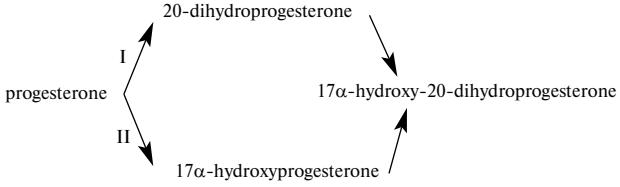


Fig. 3. HPLC analysis of progesterone transformation by the control (*S. cerevisiae* YEp51) and the transgenic (*S. cerevisiae* YEp5117α) microorganisms cultivated in YNB (*1-3*) and YPD (*4-6*) media. Progesterone (100 μM) was added at 24 h after the induction by D-galactose, and total content of extracellular and intracellular steroids was analyzed. *1*, *4*) 24-h incubation of progesterone with control strains; *2*, *3*) biotransformation of progesterone by the transgenic strain for 3 and 24 h, respectively; *5*, *6*) biotransformation of progesterone by the transgenic strain for 3 and 24 h, respectively. The absorption scale at 240 nm is 0.08 for curves *1*, *2*, *4*, *5* and 0.2 for curves *3* and *6*.

(10.9 min). While growing cells in YPD medium, in addition to 17α-hydroxyprogesterone, the biotransformation resulted in the formation of a product II with retention time of 6.8 min; its content prevailed in 24 h of the progesterone transformation (Fig. 3, curve *6*). Product II was more polar than androstendione and testosterone, but less polar than deoxycortisol. Two-dimensional TLC analysis (first direction chloroform–ether (4 : 1 v/v); second direction hexane–ether–acetone–acetic acid (8 : 15 : 1 : 1 v/v)) did not reveal either androstendione, the product of the lyase reaction C_{17}-C_{20} catalyzed by mammalian cytochrome P-45017α [2], nor testosterone, the product of androstendione transformation by the constitutive yeast 17β-steroid dehydrogenase in the ethyl-acetate extracts [21]. The products I and II were isolated and purified using TLC and HPLC for subsequent mass spectrometric analysis (Fig. 4). The mass spectrum of the standard, 17α-hydroxyprogesterone, was identical to that of product I (M^+ values at *m/z* 330, relative intensity of the peaks, and character of the fragmentation), as expected considering the substrate specificity of cytochrome P-45017α. The mass spectrum of the product II was characterized by the M^+ value of 332, and an intensity of the fragmentation peaks and character of the fragmentation differed from those parameters of presumable products of progesterone transformation by the transgenic organism: androstendione, 16-hydroxyprogesterone, and 17α,16-dihydroxy-progesterone. No changes were observed in the UV spectrum of the peak maximum after HPLC of the product II, this suggesting a conservation of the coupled Δ^4-3-keto structure of the steroid. Thus, according to HPLC, TLC, UV- and mass spectrometry studies, the product II is a steroid reduced at the 20-keto group—17α-hydroxy-20-dihydroprogesterone. The formation of a more polar metabolite than 17α-hydroxyprogesterone was revealed without its identification using a hybrid "linked" protein consisting of the heme-containing domain of bovine cytochrome P-45017α, the flavin-containing domain of rat NADPH-cytochrome P-450 reductase [28], and the cytochrome P-45017α expressed in *S. cerevisiae* under the control of the promoter and terminator of yeast alcohol dehydrogenase I [13]. It should be noted that the cytochrome P-45021 isolated from microsomes of adrenal cortex exhibited oxidase activity towards 20β-dihydroprogesterone and 17α-hydroxy-20-dihydroprogesterone [29, 30]. Reduction of keto groups of steroids at C-20 is one of the common reactions of inactivation of human steroid hormones [31]; the same reaction takes place as an undesirable side reaction of desired products in a number of industrial microbiological transformations of steroids (Δ^1-dehydrogenation, 11α-, 11β-, and 16α-hydroxylation of progesterone), which is only in partly controlled by the extent of saturation of the medium by oxygen and by the composition of the cultivation media [32].

Kinetics of progesterone transformation by the transgenic yeast *S. cerevisiae* YEp5117α while being cultivated in YNB and YPD media. Biotechnological application of recombinant microorganisms for the synthesis of 17α-hydroxylated steroids requires media providing intensive growth. Then a problem of side product formation arises due to a decrease in the specific content of the inserted mammalian cytochrome P-45017α in a total cell population. To clarify conditions and mechanisms of side product formation, we studied the kinetics of steroid transformation by the transgenic yeast while growing in YNB and YPD media (Fig. 5). The process of progesterone biotransformation by *S. cerevisiae* YEp5117α has three-phase character. An intensive uptake of progesterone from the medium without stoichiometric formation of the metabolites was observed during 0.5-1 h, the equilibrium ratio extracellular progesterone/intracellular progesterone being about 1 : 1. Thus, the first phase of progesterone transformation by transgenic microorganisms is connected with the transport of the steroid into the cells, its interaction with cytochrome P-45017α localized in the membranes of endoplasmic reticulum, and preliminary stages of the monooxygenase reaction (induction of the high-spin form of the cytochrome, interaction with the yeast constitutive NADPH-dependent cytochrome P-450 reductase, etc.). These data are confirmed by the spectrophotometric titration of the cell culture by progesterone: a maximal spectral response of type I ($\Delta A_{390\ nm} - A_{420\ nm}$) was observed in 5-10 min after the addition of progesterone. For the subsequent 1-5 h (the second phase), the progesterone uptake became slower, and a virtually proportional increase of 17α-hydroxyprogesterone in the outer medium was observed, reaching its maximal value in 10-23 h (the third phase). During this steady-state phase, 17α-hydroxylation of the progesterone molecule and its excretion from the cell take place (Fig. 5a). Maximal yields of 17α-hydroxyprogesterone were 80 and 45% for YNB and YPD media, respectively. The differences in the yields of the purpose product was due to the fact that after 2-3 h of incubation, 17α-hydroxy-20-dihydroprogesterone formation was detected in the incubation mixture, and its content increased linearly until 23 h of incubation and was more pronounced for the transgenic yeast cultivated in YPD medium (Fig. 5b). This product can be formed in the following two ways:



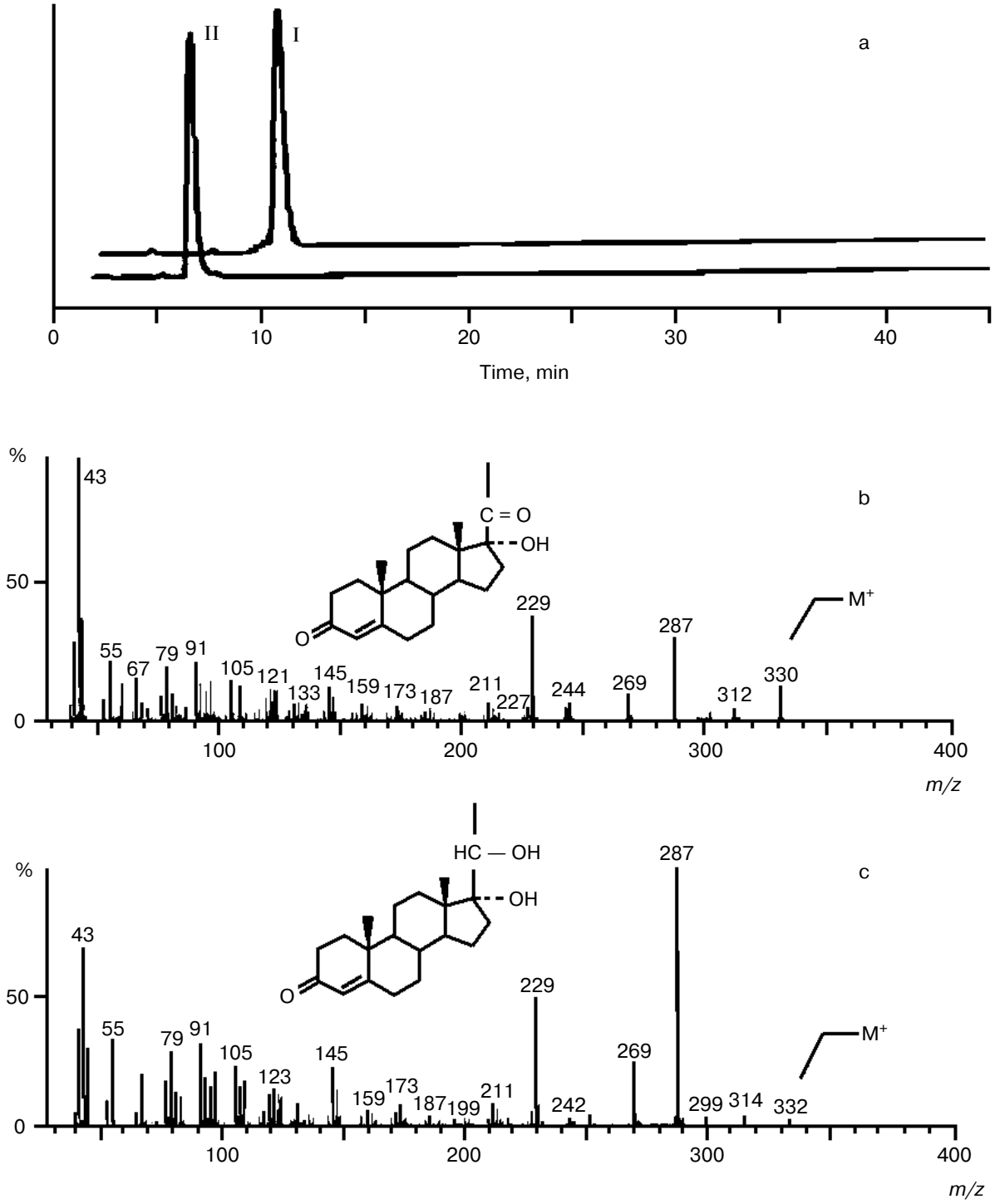


Fig. 4. HPLC analysis of the purity (a) and mass spectra of the products I (b) and II (c) isolated from the supernatants of cell cultures after 24-h biotransformation of progesterone.

The kinetics of the accumulation of the products (Fig. 3) indicates that the reaction proceeds via pathway II.

Figure 6 illustrates progesterone consumption from the culture medium, the formation of 17α-hydroxyprogesterone and 17α-hydroxy-20-dihydroprogesterone after the simultaneous addition of the substrate and the inducer of the cytochrome P-45017α synthesis to the cultivation media. The process of the accumulation of the steroid products exhibited a pronounced lag-phase of 5-8 h, which was accompanied by

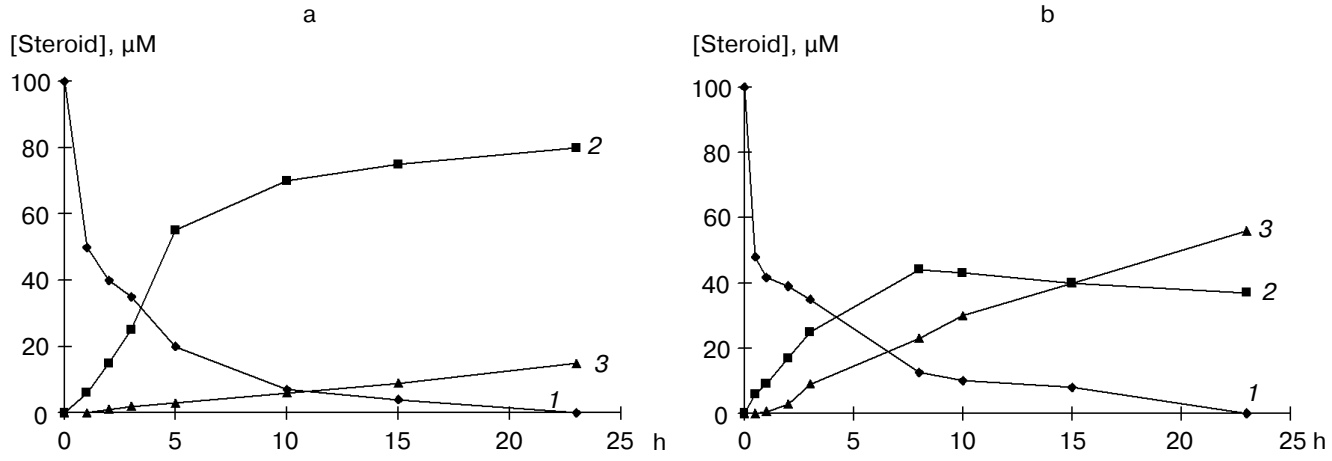


Fig. 5. Changes in steroid concentrations in the extracellular medium during progesterone biotransformation by *S. cerevisiae* YEp5117α. Progesterone was added to the culture 24 h after the induction of the cytochrome P-45017α synthesis by D-galactose: a) cultivation in YNB medium; b) cultivation in YPD medium. Concentration of the substrate was 100 μM. *1*) Progesterone; *2*) 17α-hydroxyprogesterone; *3*) 17α-hydroxy-20-dihydroprogesterone. Each point of the curves is the mean value of two independent experiments.

a low level of progesterone biotransformation but intensive consumption of the original substrate by the microorganisms. The lag-phase corresponded to the period of induction of cytochrome P-45017α synthesis, this being confirmed by the similar kinetics of the heme protein accumulation monitored by the changes in CO difference spectra. After the induction stage, the extent of the substrate transformation into 17α-hydroxyprogesterone sharply increased with subsequent formation of 17α-hydroxy-20-dihydroprogesterone. It has been shown that 17α-hydroxyprogesterone is actively excreted by the transgenic yeast into the extracellular medium, although a part of the 17α-hydroxyprogesterone pool remains in the cells due to the equilibrium (the ratio extracellular 17α-hydroxyprogesterone/intracellular 17α-hydroxyprogesterone is 80-85% to 15-20%). As a result, 17α-hydroxyprogesterone is reduced inside the cells, yielding 17α-hydroxy-20-dihydroprogesterone, which is almost completely excreted from the cells.

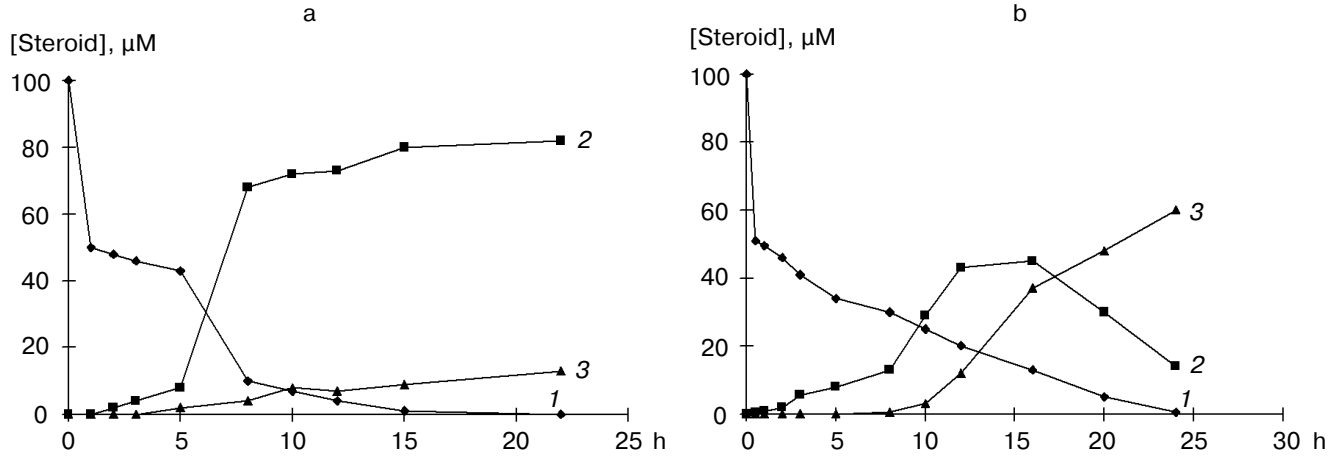


Fig. 6. Changes in concentrations of steroids in the extracellular medium during the biotransformation of progesterone by *S. cerevisiae* YEp5117α. Progesterone and D-galactose were added into the culture medium simultaneously: a) cultivation in YNB medium; b) cultivation in YPD medium. Concentration of the substrate was 100 μM. *1*) Progesterone; *2*) 17α-hydroxyprogesterone; *3*) 17α-hydroxy-20-dihydroprogesterone. Each point of the curves is the mean value of two independent experiments.

Effect of modifiers of steroid biosynthesis and monohydroxylated progesterone derivatives on the 17α-hydroxylase and 20-reductase activities of the transgenic microorganisms. The assumption that the synthesis of 17α-hydroxyprogesterone is catalyzed by the inserted cytochrome P-45017α and the formation of 17α-hydroxy-20-dihydroprogesterone is due to the functioning of the constitutive 20-ketosteroid reductase was confirmed by the experiments on the transformation of progesterone and 17α-hydroxyprogesterone in the presence of modifiers of steroid synthesis (metirapon, dexamethasone, CO). Metirapon (25 μM) and dexamethasone (100 μM) do not affect the relative yields of 17α-hydroxyprogesterone and 17α-hydroxy-20-dihydroprogesterone while using progesterone as the substrate compared to the experiments in the absence of these compounds. Carbon monoxide does not affect the 20-ketosteroid reductase activity towards 17α-hydroxyprogesterone and sharply inhibits 17α-hydroxylation of progesterone and coupled with this reaction subsequent 20-reduction of 17α-hydroxyprogesterone. To clarify the relationship between the functioning of cytochrome P-45017α and the 20-ketosteroid reductase, we used a number of monohydroxylated progesterone derivatives (table) as the substrates for transformation. The steroid products were separated and their yields were estimated using HPLC analysis. The potential products of the 17α-hydroxylation and 20-ketoreduction reactions were estimated by the increase of M^+ in the mass spectra by 16 and 2, respectively. As an example of these experiments, Fig. 7 presents the HPLC chromatogram of the product of 11β-hydroxyprogesterone biotransformation by the transgenic strain and its mass spectrum. The UV spectrum of the product with the retention time of 4.0 min (yield 37.2% for 24 h) exhibited no changes (the absence of reduction at the Δ^4-bond, and the molecular ion peak was 346, this indicating 17α-hydroxylation of 11β-hydroxyprogesterone, according to the substrate specificity of cytochrome P-45017α. 11α-Hydroxyprogesterone was also subjected to 17α-hydroxylation with a relatively low efficiency (yield of 11α,17α-dihydroxyprogesterone was 31.4% for 24 h of transformation). It was shown that 21-hydroxyprogesterone (deoxycorticosterone) was transformed with a lower yield (11.7% for 24 h) into 21-hydroxy-20-dihydroprogesterone without formation of 17α,21-dihydroxyprogesterone (deoxycortisol), since the retention time for deoxycortisol under the conditions employed was 4.5 min, and the mass spectrum of the product indicated the 20-reduction. These data can be explained considering the substrate specificity of cytochrome P-45017α from adrenal cortex microsomes that determines the original 17α-hydroxylation with the subsequent 21-hydroxylation of steroids by cytochrome P-45021 in the course of biosynthesis of glucocorticoids [1]. 19-Hydroxyprogesterone was not subjected to either 17α-hydroxylation or 20-reduction; minor products (2% yield for 24 h) were not identified in the present work because of their insufficient amount. 17α-Hydroxyprogesterone is effectively reduced at the 20-keto group, yielding 91-97% of the reduced product for 24 h of transformation by both the recombinant and the control microorganisms. Thus, the appearance of the 17α-hydroxyl group as a result of progesterone hydroxylation is of critical importance for the subsequent 20-ketosteroid reductase activity. This can be summarized as the following series of relative substrate efficiency in the biotransformation reactions:

17α-hydroxylation: progesterone > 11β-hydroxyprogesterone > 11α-hydroxyprogesterone > 19-hydroxyprogesterone;

20-ketoreduction: 17α-hydroxyprogesterone > 21-hydroxyprogesterone > 19-hydroxyprogesterone.

Figure 8 illustrates processes of progesterone consumption by the cells of the transgenic microorganisms, its subsequent transformation, and excretion of the resulting products from the cells. Progesterone (I) is actively consumed by the cells. The first product, 17α-hydroxyprogesterone (II), is distributed between cell and extracellular medium according to the equilibrium, this suggesting that 80-85% of the steroid is excreted from the cell to the medium. The intracellular 17α-hydroxyprogesterone is then reduced at the 20-keto group by the 20-ketosteroid reductase yielding 17α-hydroxy-20-dihydroprogesterone (III). This product, being the most polar one, is almost completely excreted from the cells without the C_{17}-C_{20} lyase reaction or other transformations. We can assume that the ratio between the 17α-hydroxy- and the 17α-hydroxy-20-dihydroprogesterone derivatives is determined by the level of expression of cytochrome P-45017α, which can exhibit the oxidative activity towards the reduced at the 20-keto group substrates similarly to cytochrome P-450scc (oxidase activity towards testosterone [33]) and P-45021 (oxidase activity towards 20-dihydroprogesterone or 17α-hydroxy-20-dihydroprogesterone [29, 30]). A decrease in the specific content of cytochrome P-45017α in YPD medium results in prevailing of the 20-ketosteroid reduction compared to the 20-OH oxidation; therefore, the side product is a minor product in selective YNB medium and a predominant product during cultivation in YPD medium.

The 17α-hydroxylation reaction is one of the most important steps in the process of synthesis of prednisolone and its more active analogs, components of well known medicines. It should be noted that the reaction of 17α-hydroxylation is not used in biotechnology, since patent strains of natural microorganisms exhibit low functional activity with 20% yield of 17α-hydroxyprogesterone, also possessing side hydroxylating activities towards the 6β-

Biotransformation of progesterone and its monohydroxylated derivatives by the control (*S. cerevisiae* YEp51)* and the recombinant (*S. cerevisiae* YEp5117α)** microorganisms (YPD medium, induction by D-galactose for 24 h)

Substrates (retention time by HLPC, min)	Time of biotransformation, h	Products			
		retention time by HPLC, min	yield, %	M^+, *m/z*	
Progesterone* (32.3)	24	32.3	100	314	
Progesterone** (32.3)	6	32.3	14		
		10.9	57	330	[a]
		6.8	29	332	[b]
	24	10.9	38.3		
		6.8	61.7		
17α-Hydroxyprogesterone* (10.9)	6	10.9	28.0	330	[a]
		6.8	72.0	332	[b]
	24	10.9	2.8		
		6.8	97.2		
17α-Hydroxyprogesterone** (10.9)	6	10.9	40.8	330	[a]
		6.8	59.2	332	[b]
	24	10.9	9.0		
		6.8	91.0		
11β-Hydroxyprogesterone** (9.6)	6	9.6	88.3		
		4.0	11.7		
	24	9.5	62.7	330	
		4.0	37.2	346	[c]
11α-Hydroxyprogesterone** (5.8)	6	5.8	86.4		
		3.0	13.6		
	24	5.8	68.6	330	
		3.0	31.4	346	[d]
21-Hydroxyprogesterone** (9.7)	6	9.7	95.4		
		5.7	1.7		
		5.3	2.9		
	24	9.6	85.6	330	
		5.7	2.6		
		5.3	11.7	332	[e]
19-Hydroxyprogesterone** (6.6)	6	6.6	100		
	24	6.6	96.0		
		5.7	2.0		
		4.1	2.0		

[a] 17α-Hydroxyprogesterone.
[b] 17α-Hydroxy-20-dihydroprogesterone.
[c] 11β,17α-Dihydroxyprogesterone.
[d] 11α,17α-Dihydroxyprogesterone.
[e] 21-Hydroxy-20-dihydroprogesterone.

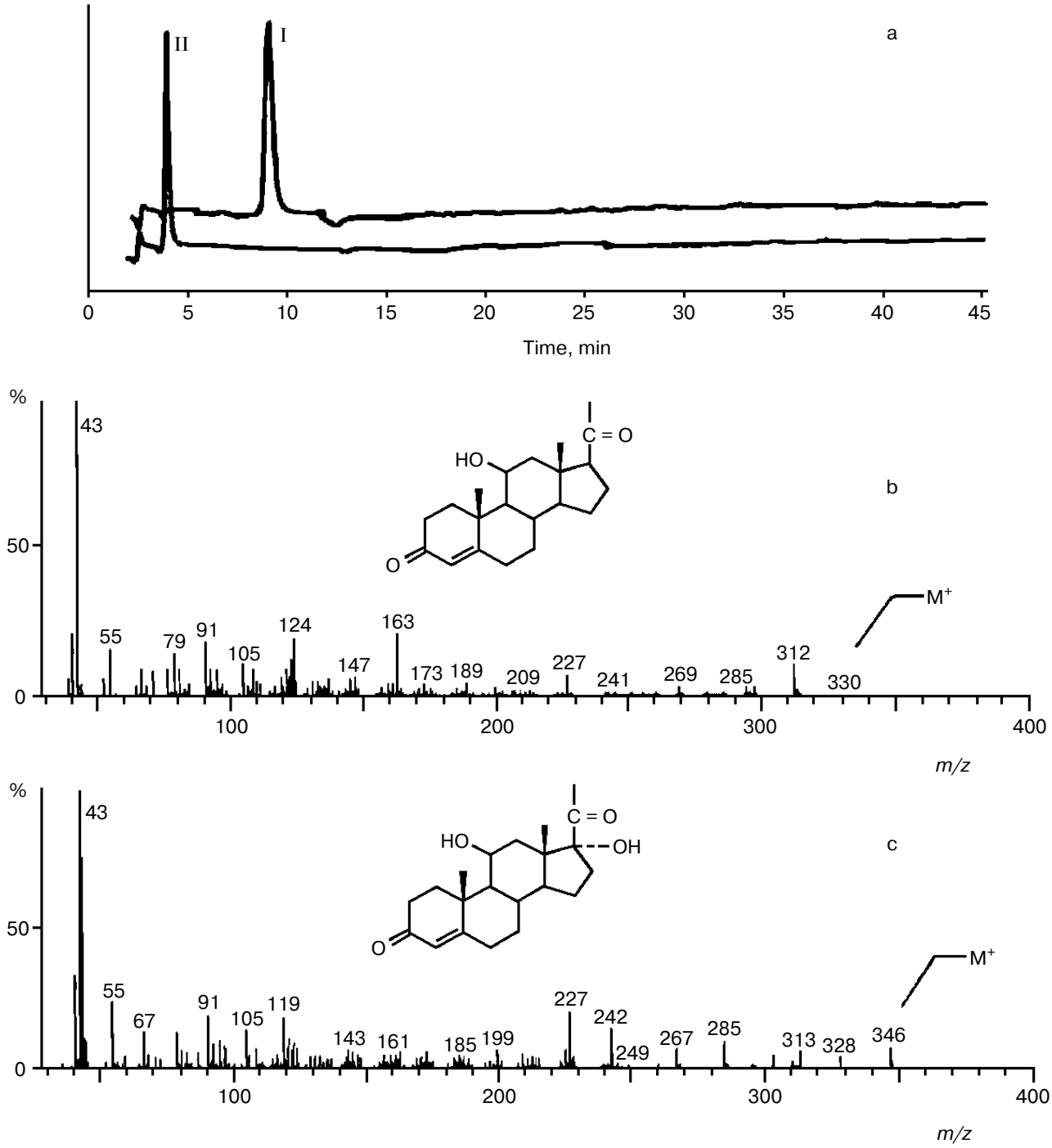


Fig. 7. HPLC analysis of the purity extent (a) and mass spectra of 11β-hydroxyprogesterone (b) and the product of its biotransformation (c).

and the 11α-positions [34]. High yield of the desired product obtained in the present work is a good background for the biotechnological synthesis of 17α-hydroxylated steroids. Being an undesirable side product of the biotechnological process, 17α-hydroxy-20-dihydroprogesterone is of great importance for chemico-enzymatic synthesis of compounds inhibiting both 17α-steroid hydroxylase/C_{17}-C_{20} lyase and 5α-steroid reductase, the enzymes playing the leading role in the formation and growth of benign and malignant tumors of human prostate.

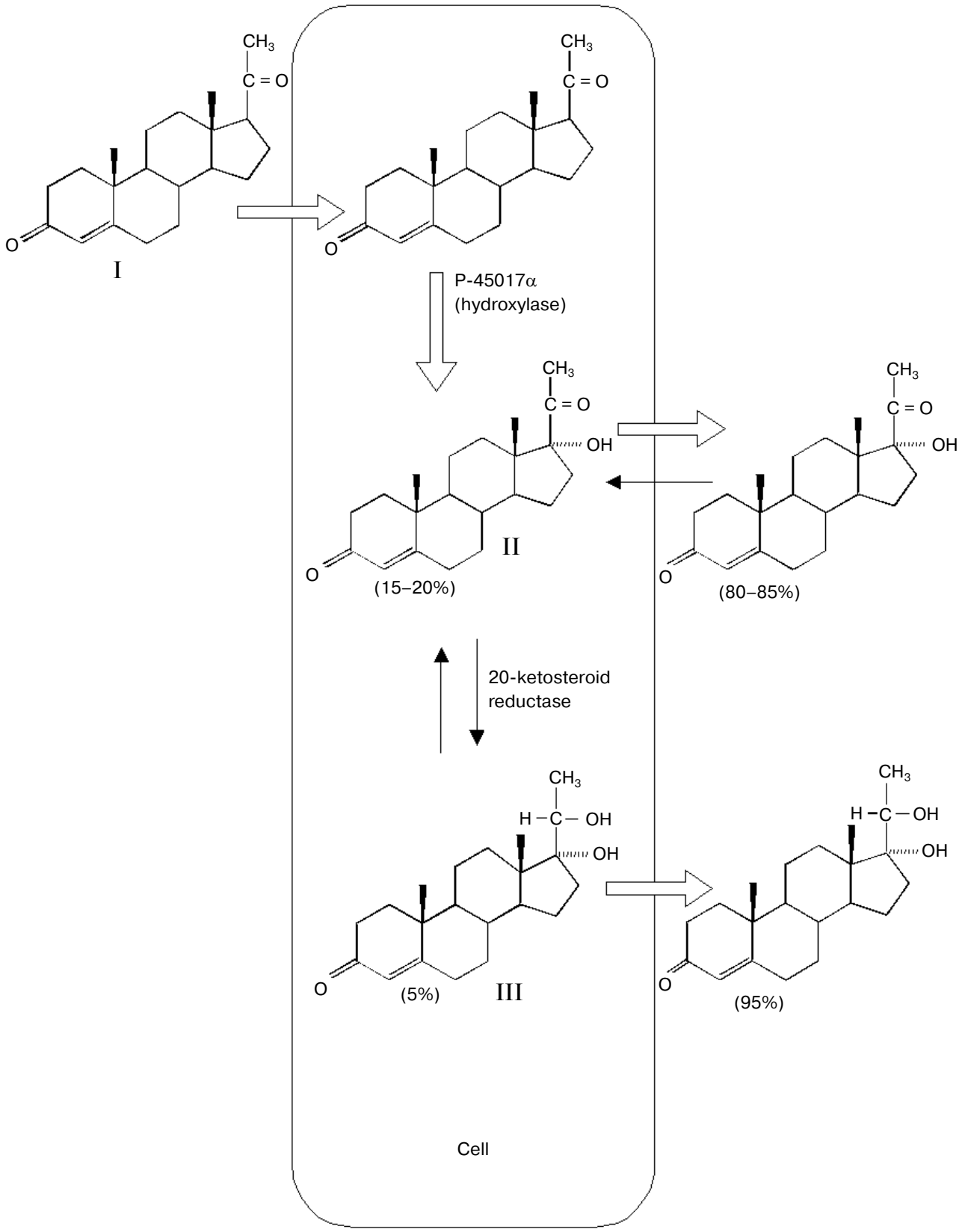


Fig. 8. Scheme of progesterone consumption by a cell of transgenic microorganism, its enzymatic transformation, and excretion of steroid products into the extracellular medium.

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