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A Prospective Study
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Urine Contamination by micro-organisms and alterations in the endogenous steroid profile. A prospective study.

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Introduction

Among the substances controlled in doping control, there is the prohibited class of anabolic agents (following IOC classification), including androgenic anabolic steroids (AAS). The detection of ASS consumption is routinely performed in urine specimens by gas chromatography coupled to mass spectrometry (Ward R., 1976; Donike M., 1984; Segura J., 1993). The screening for these compounds, includes either the detection of synthetic AAS (i.e. stanazolol, methyltestosterone), synthetic natural AAS (i.e. dihydrotestosterone, testosterone) or their metabolic precursors (dehydroepiandrosterone and androstendione) and the evaluation of the natural steroid profile (i.e. androsterone, etiocholanolone, 5α and 5β androstandiols and androstandiones) (Southan GJ., 1992 and Donike M., 1993). Laboratories involved in doping control have been always concerned by the degradation of steroids by micro-organisms after several *in vitro* (Geyer H., 1996) and *in vivo* observations (Catlin DH., 1996 and Schänzer W., 1995). In fact these observations are not surprising since micro-organisms have been used for years in the synthesis of some steroids because the ability of some bacterial enzymes of catalysing several chemical reactions (Heftmann E. 1968). Then, when present in a biological fluid like urine some of these chemical reactions may occur, modifying substrates present, namely endogenous or exogenous steroids. The origin of such micro-organisms can be easily explained in several ways: their presence in the collected urine, in the non sterile material used for urine collection, and finally uncontrolled sample contamination on manipulation during analysis or storage at the laboratory.

Up to date several studies have been focused in the identification of markers of micro-organisms metabolic activity over urinary excreted steroids like 5α-androstandione, 5β-androstandione and androstendione usually present in urine at very low concentrations but which concentrations rise very quickly in contaminated urine (Ayotte C., 1996, Greyer H.,

1996). Some authors have tried to identify micro-organisms potentially involved in alterations of the urinary endogenous steroid profile. Those studies have been performed either in urine specimens already showing signs of bacterial contamination (Ayotte C., 1996) or left to be contaminated spontaneously by environmental micro-organisms (Hemmersbach P., 1996). The main objective of this work is to perform a prospective controlled study of urine contamination focusing on alterations induced by selected micro-organisms in the urinary endogenous steroid profile. Selection of micro-organisms was performed choosing species involved commonly in urinary infection in humans, some belonging to the normal flora and several that can be classified as laboratory contaminants.

Materials and Methods

Reference steroids

Non deuterated reference steroids and methyltestosterone (MT) were purchased from Sigma (St. Louis, USA). Testosterone-[16,16,17]-d_3 (Td3), 17 -epitestosterone-[16,16,17]-d_3 (Ed3) and etiocholanolone-[2,2,4,4]-d_4 (Etd4) were kindly provided by the late Prof. M. Donike (DSHS, Cologne, Germany).

Reagents

The derivatization reagent N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA) was provided by Macherey-Nagel (Düren, Germany), Ammonium iodide GR was from Merck and dithioerythritol 99+% from Aldrich-Chemie (Steinheim, Germany) were used as supplied. The enzyme β-glucuronidase from *E. coli* (K12) was provided by Boehringer Mannheim (Germany). Detectabuse columns (XAD2) were provided by Biochemical Diagnostics Inc. (NY, USA). All other reagents were of analytical grade and used as supplied.

Sample

A pooled urine from a healthy male volunteer was collected and sterilised by filtration (0.22 μm) following a procedure previously described (Segura J., 1989). Urine aliquots (15 ml) were stored in sterile glass tubes. Previous studies of this volunteer showed a standard steroid profile as compared with reference values of the Caucasian population (Donike M. 1995 and Ayotte C., 1996). Aliquots were inoculated with known amounts of 15 different micro-organisms (bacteria, yeast and moulds) (see Table 1).

Microbiological controls

Sterility control

Before micro-organisms inoculation, sterile urine aliquots were submitted to a sterility control. This control consisted on inoculating 100 µl of urine in 10 ml culture medium thioglicolate and 10 µl in blood agar, Sabouraud, Gardnerella and MacConkey agar plates. Cultures were incubated 14 days. Controls were performed at 24 and 48 hours and 7 and 14 days.

Micro-organisms growth

Micro-organisms growth in urine was controlled just after their inoculation, 24 hours later (growing log phase) except for moulds (96 hours) and finally after two weeks of incubation at 37°C. In each growing stage, 10 ml of urine were separated and kept frozen (-20°C) until chemical analysis. The remaining urine volume was submitted to serial dilutions with physiologic serum until reaching 10000 CFU/ml (CFU stands for colonies forming units). The undiluted urine and 100 µl of each dilution were inoculated in the corresponding agar culture plates described previously. Plates where the number of CFU ranged between 30 and 90 were the ones selected for counting. Counting was performed in duplicate.

Urease activity test

For the characterisation of micro-organisms evaluated, urease activity was tested culturing the fungi in Christensen medium, *P. aeruginosa* in Bacto urea agar base (Difco^{R}). For cocci and bacilli Gram positive the test Api^{R} Coryne was utilised. For *enterobacteriaceae* the urease test Api ^{R} 20 E was used.

Urinary Steroid profile analysis

Urine samples obtained at pre-inoculation, day one (4 for moulds) and day 15 were analysed using standard screening procedures for anabolic agents (free and conjugated fractions were analysed independently) (Segura 1993). Testosterone (T), epitestosterone (E), androsterone (A), etiocholanolone (Et), 5α-androstan-3α,17β-diol (5 -DIOL), 5β-androstan-3α,17β-diol

(5 -DIOL), 5α-androstandione, 5β-androstandione, androstendione and dehydroepiandrosterone (DHEA) were monitored in each urine specimen.

Results and Discussion

Results concerning micro-organisms growth are presented in Table 1. As expected bacterial growth was nearly stopped in urine samples after 15 incubation days while after 24 hours of the inoculation, is in its logarithmic phase. Moulds that have a slower cycle of life were still present in the samples in considerable amounts after two weeks of incubation. All sterility controls showed not growing of any micro-organism demonstrating that all urine aliquots before inoculation were sterile. Microbiological controls in the negative control (non-inoculated, but incubated) showed no growing of micro-organisms in this urine.

The comparison of control urine samples, the one frozen immediately after urine sterilisation and the non-inoculated one but incubated at 37°C for two weeks in parallel with inoculated urine, showed that to some extend conjugated steroids are de-conjugated spontaneously in the experimental conditions tested. Regarding sulphate conjugates, DHEA-sulphate appears to be more labile than glucuronides. No other significant chemical modifications of steroids were observed (see Table 3) in the control urine samples. By comparing steroids concentrations in the free and conjugated fractions of inoculated samples to their corresponding control sample (day 1 and 15), it can be observed that several micro-organism studied induced a de-conjugation of steroids.

In the present somehow extreme conditions of the study, such criterion has not been fully confirmed (see Figure 1). For most of the micro-organisms tested, particularly after two weeks incubation hydrolysis of glucuronides of T and/or E were higher than 5%. Nevertheless, the non-inoculated incubated control samples also showed rates of de-conjugation slightly higher than 5% but lower than 10%. It seems then that in addition to well accepted indicators (i.e. 5α-androstandione, 5β-androstandione and androstendione) the presence of non-conjugated endogenous steroids over 10% is one of the best markers of micro-organisms urine contamination or urine exposition to high temperatures. The rate of steroid glucuronides de-conjugation highly depends also on the configuration of the steroid chemical structure. For

example T and E conjugates are more labile to hydrolysis than the corresponding metabolic products A and Et, being Et conjugates more labile than those of A (see Figure 2).

Sulphate conjugates stability depends on urinary pH changes induced by micro-organisms growth. They are apparently more stable in alkaline conditions (See Figure 3). Changes in urinary pH are strongly linked to urease activity of the micro-organism. When urease activity is high, pH is high and then the recovery of urinary free DHEA is lower. Globally, and regarding urinary pH, it has been assumed for a long time that alkaline urinary pH was associated to urine bacterial contamination. Present results does not support this view as many micro-organisms (those urease negative) does not induce significant changes in urinary pH but several metabolic changes, including de-conjugation of glucuronides and sulphates, in the endogenous steroid profile.

Regarding changes produced by micro-organisms in typical markers of urine contamination, *C. jekeium* induced relevant changes in 5α-androstandione, 5β-androstandione and androstendione urinary concentrations as compared to the other micro-organisms studied (see Figure 4). The evaluation of the T/E ratio in doping control is at present the sole official parameter to detect testosterone abuse among athletes. Its artifactual modification may lead to misinterpretation of results. In vitro synthesis of T has been already described in the sample preparation process. *De novo* synthesis of testosterone or epitestosterone was not detected for any of the micro-organisms studied. Nevertheless, in a non controlled situation like in routine doping control even if it is unlikely, the synthesis of T and E cannot be discarded.

Conclusions.

Alkalisation of urinary pH is not a good indicator of urine contamination as several micro-organisms can growth in this medium without any noticeable alteration of this parameter. In non contaminated urine steroid glucurono-conjugates are hydrolysed spontaneously to a low extend (less than 10%). Higher hydrolysis rates were observed for DHEA-sulphate. In all contaminated urine samples de-conjugation was the major observation even after 24 hours incubation. Being at present this observation, one of the most consistent in all micro-organisms tested. A non-conjugated fraction higher than 10% of the total amount of T is a good indicator of sample degradation. There is no production of T or E after micro-organisms growth in urine samples. In addition some micro-organisms are able to synthesise 5 -

androstandione, 5 -androstandione and androstendione using endogenous steroids as substrates. With the present analytical approach and current positivity criteria it is not possible to release any positive result for T, DHT and any T metabolic precursor (DHEA, androstendione, androstendiol) when signs of urine contamination are evident. Most probably the only analytical technique available that is able to overcome this situation will be gas chromatography coupled to isotope ratio ($^{13}C/^{12}C$) mass spectrometry (GC/C/IRMS).

Table 1. Micro-organisms evaluated and growing pattern in culture media

MICRO-ORGANISMS	Urease activity	inoculum[1]	Initial Counting[2] (0h)	Log Phase[2]	Counting[2] After 15 Days
Fungi and Moulds					
Candida albicans	-	0.2*	2.3x10E5	6.5x10E6	2.0x10E6
Penicillum sp.	+	0.5 **	1.5x10E5	1.0x10E5	2.7x10E4
Aspergillus flavus	+	0.5 **	1.0x10E6	1.0x10E6	2.4x10E5
Bacteria					
Staphylococcus epidermidis	+	0.5	8.0x10E5	1.0x10E7	3.0x10E3
Pseudomonas aeruginosa	+	0.05	2.3x10E6	1.5x10E8	3.0x10E6
Escherichia coli	-	0.05	8.0x10E5	7.0x10E7	1.2x10E5
Klebsiella pneumoniae	+	0.05	7.0x10E5	5.3x10E7	2.7x10E2
Proteus mirabilis	+	0.05	9.0x10E5	3.0x10E7	< 10
Enterococcus faecalis	-	0.5	6.0x10E6	1.2x10E8	2.3x10E6
Corynebacterium sp.	+	0.45*	7.0x10E6	8.9x10E7	3.0x10E1
Corynebacterium jekeium	-	0.45*	3.0x10E2	8.0x10E5	2.0x10E2
Streptococcus mitis	-	0.45*	2.0x10E5	4.0x10E6	< 10
Streptococcus agalactiae	-	0.45*	1.0x10E6	1.0x10E7	< 10
Dödderleïn bacill	-	0.6*	6.5x10E5	1.5x10E6	< 10
Gemella hemolis ans	-	0.45*	7.0x10E5	5.0x10E6	< 10
Negative Control (urine non contaminated)			< 10	< 10	< 10

[2] counting in ufc/ml

[1] thioglicolate broth (ml) for a final concentration ca. 10^5 ufc/ml

* broth after 48 hours invcubation

**suspension in physiologic serum plus Tween 20

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Figura. 1

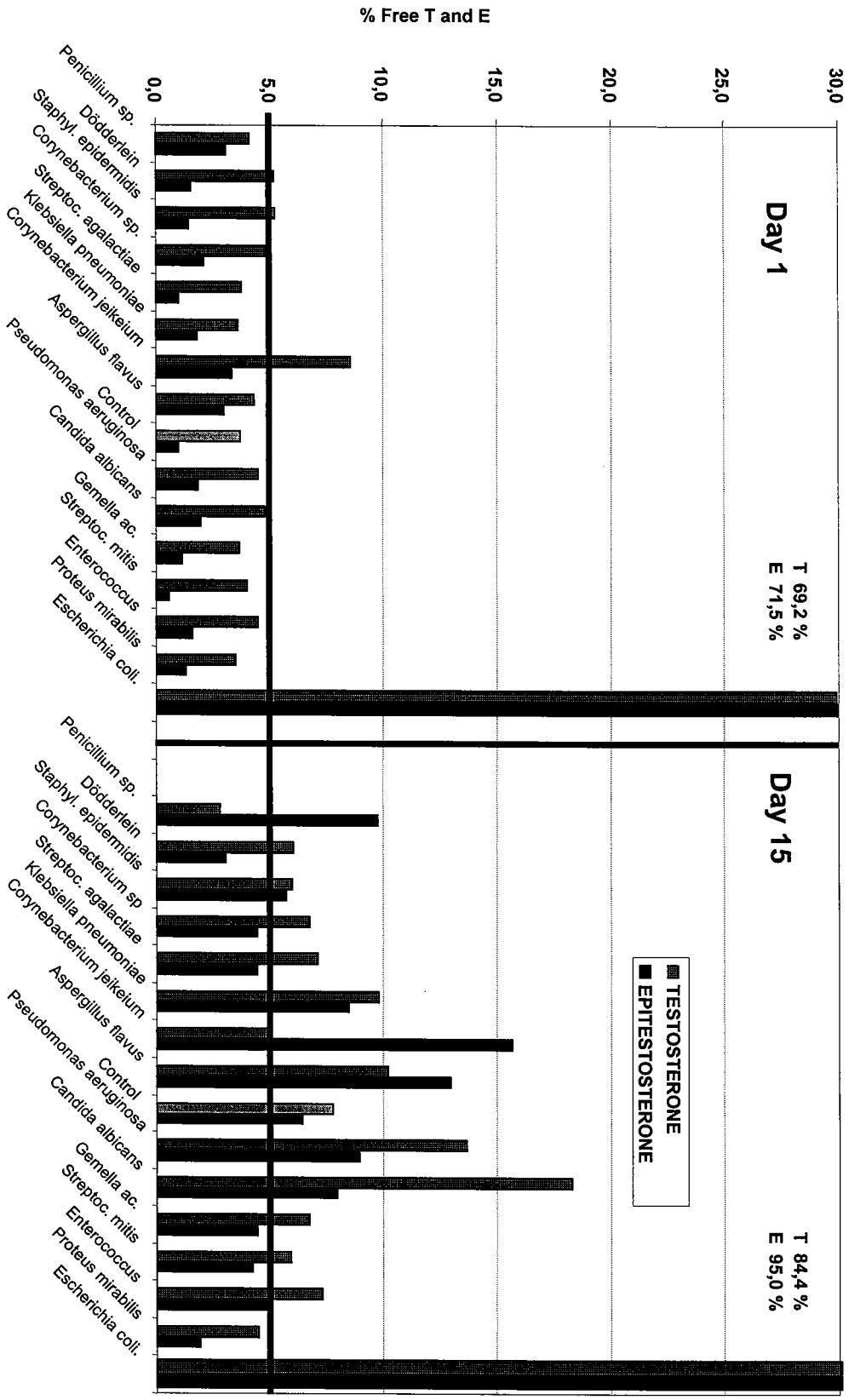

Testosterone and Epitestosterone - Free Fraction
% Free T and E
30,0
25,0
20,0
15,0
10,0
5,0
0,0
Day 1
T 69,2 %
E 71,5 %
Day 15
T 84,4 %
E 95,0 %
TESTOSTERONE
EPITESTOSTERONE
Penicillium sp.
Dödderlein
Staphyl. epidermidis
Corynebacterium sp.
Streptoc. agalactiae
Klebsiella pneumoniae
Corynebacterium jeikeium
Aspergillus flavus
Control
Pseudomonas aeruginosa
Candida albicans
Gemella ac.
Streptoc. mitis
Enterococcus
Proteus mirabilis
Escherichia coli.

Figure 2.

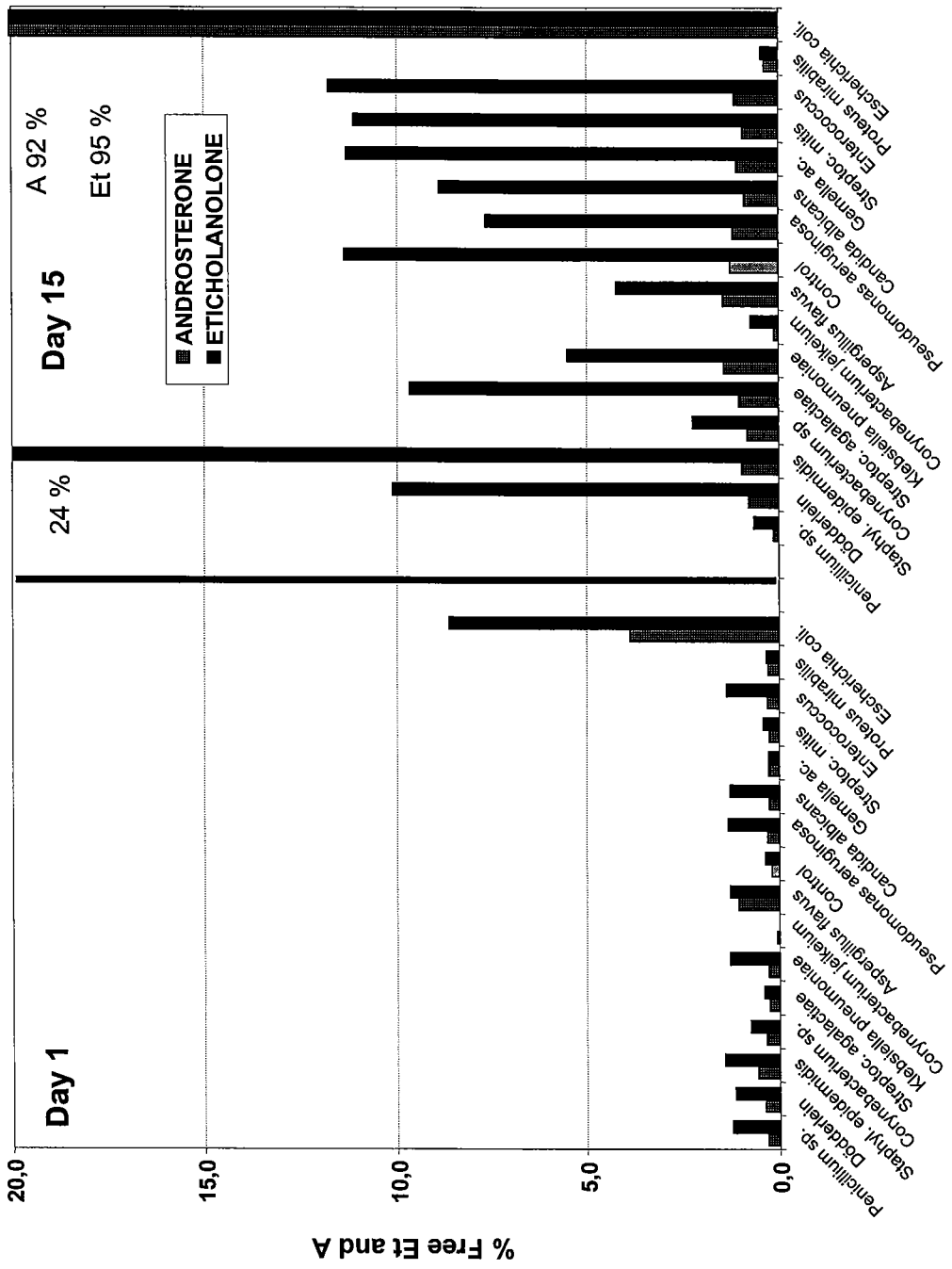
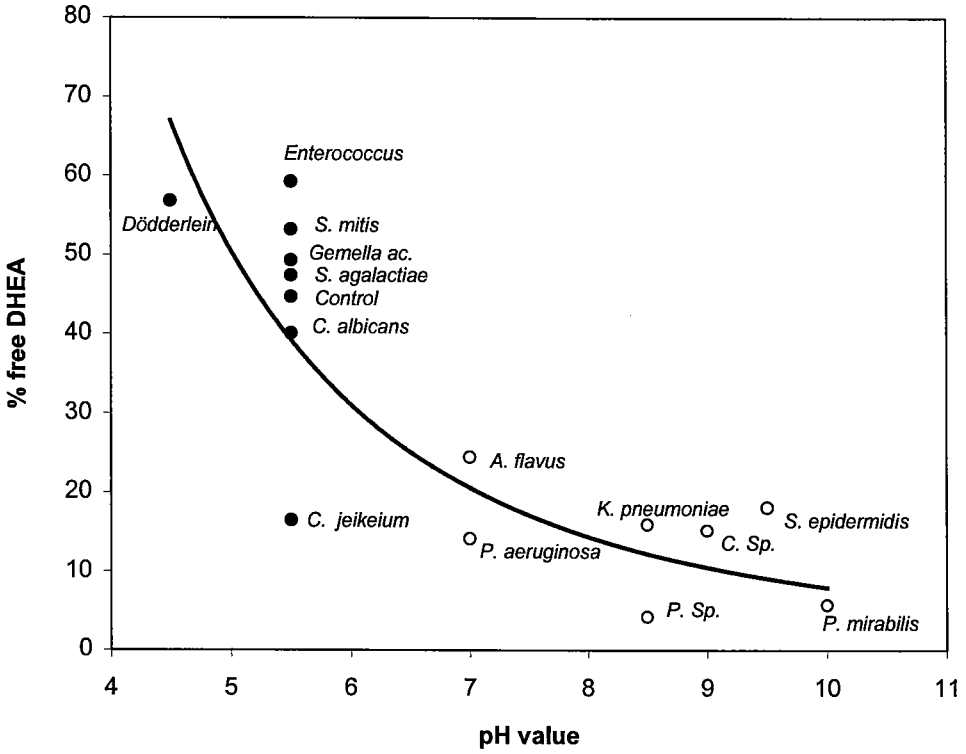

Androsterone and Etiocholanolone - Free Fraction
Day 1
24 %
Day 15
A 92 %
Et 95 %
ANDROSTERONE
ETICHOLANOLONE
% Free Et and A
20,0
15,0
10,0
5,0
0,0
Penicillium sp.
Doddemeln
Staphyl. epidermidis
Corynebacterium sp
Streptoc. agalactiae
Klebsiella pneumoniae
Corynebacterium jeikeium
Aspergillus flavus
Control
Pseudomonas aeruginosa
Candida albicans
Gamella ac.
Streptoc. mitis
Enterococcus
Proteus mirabilis
Escherichia coli.

Figure 3. Free DHEA percentage *vs* urinary pH



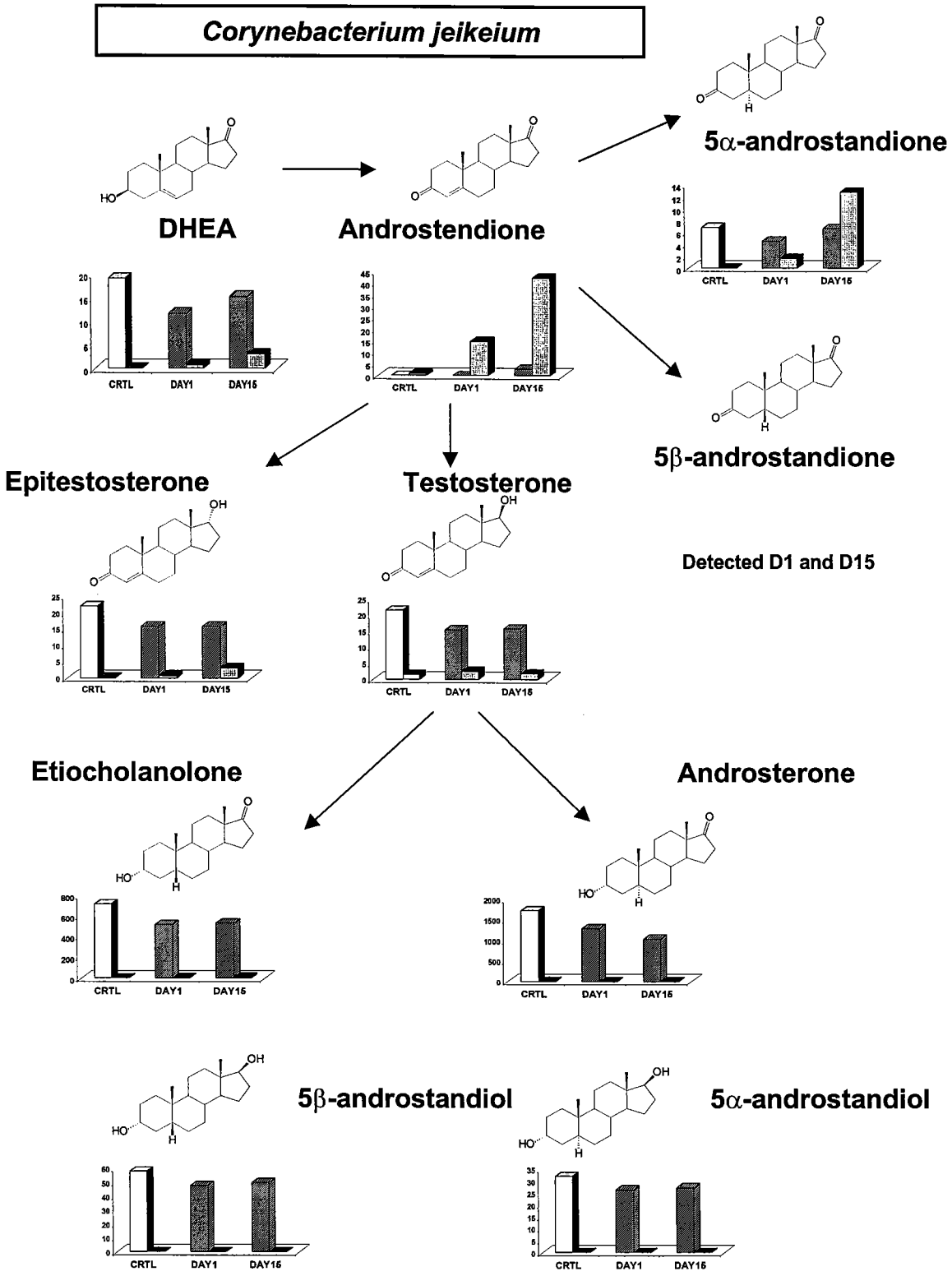

Corynebacterium jeikeium
DHEA
Androstendione
5α-androstandione
5β-androstandione
Epitestosterone
Testosterone
Detected D1 and D15
Etiocholanolone
Androsterone
5β-androstandiol
5α-androstandiol
CRTL
DAY1
DAY15

Figure 4.