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REDUCTION OF ALDOSTERONE BY ANAEROBIC BACTERIA: ORIGIN OF URINARY 21-DEOXY METABOLITES IN MAN

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SUMMARY

Aldosterone was incubated with pure cultures of human faecal micro-organisms and the products were analysed as methyloxime/trimethylsilyl ethers by gas chromatography/mass spectrometry. *Clostridium paraputrificum* quantitatively reduced the A-ring of aldosterone to a 3α-hydroxy-5β group (tetrahydroaldosterone). In addition, aldosterone was quantitatively 21-dehydroxylated by culture no. 116 and *Eubacterium lentum*. Metabolism of aldosterone with mixed faecal flora yielded a single metabolite, 21-deoxy-tetrahydroaldosterone, reflecting the enzymatic action of both *C. paraputrificum* and culture no. 116. It is likely, therefore, that the 21-deoxymetabolites of aldosterone which have been identified in urine are formed by the action of these organisms during the passage of tetrahydroaldosterone through the gut following biliary excretion. The reduced metabolites are reabsorbed prior to urinary excretion. The specificity and high yield of the reactions carried out by these organisms suggest that they will prove useful for small scale production of reference compounds not yet commercially available, since almost all steroids with α-ketol side chain have been shown to be susceptible to transformation.

INTRODUCTION

Only about 50–55% of aldosterone administered to man is excreted as tetrahydroaldosterone glucuronide, acid-labile 18-oxo-glucuronide and free aldosterone; a large fraction is shared by other metabolites and derivatives of these metabolites [1].

Amongst the metabolites identified in urine by Kelly and associates [1–4] following aldosterone administration, are steroids without a hydroxyl group at position 21: 21-deoxytetrahydroaldosterone (Fig. 1, I) and a 21-deoxybicyclic acetal (II). It has been suspected for many years that 21-dehydroxylation, an important catabolic transformation in man, is not a reaction carried out by mammalian enzyme systems and evidence has been obtained that the intestinal microflora carry out this reduction following biliary excretion of steroids [5]. We have recently isolated for the first time a bacterial organism capable of 21-dehydroxylation and have shown that it successfully reduced deoxycorticosterone (DOC), tetrahydro DOC and other steroids with α-ketol side chain [6–10]. We have therefore attempted the microbial synthesis of 21-deoxymetabolites of aldosterone to offer circumstantial evidence for the enterobacterial formation of those 21-deoxymetabolites isolated by Kelly and co-workers.

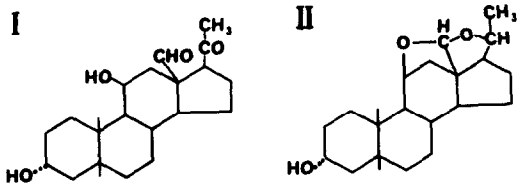

Fig. 1. Structure of 21-deoxy metabolites of aldosterone identified in urine.

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The following non-standard abbreviations have been used: *Tetrahydroaldosterone*-3α, 11β, 21-trihydroxy-20-oxo-5β-pregnan-18-al; *21-deoxyaldosterone*-11β-hydroxy-3, 20-dioxo-4-pregnan-18-al; *21-deoxytetrahydroaldosterone*- 3α, 11β-dihydroxy-20-oxo-5β-pregnan-18-al; *dehydroxylation*—substitution of a hydroxy group by a hydrogen atom.

MATERIALS AND METHODS

Aldosterone was purchased from Steraloids, Inc., Wilton, NH.

Solvents. The solvents were reagent grade except for methanol, which was technical grade.

Media. Prereduced Brain Heart Infusion Broth supplemented with 0.05% cysteine HCl (PR) was obtained from Scott Laboratories, Inc., Fiskeville, RI. Prepared thioglycollate broth (50 ml/vial) and supplemented Peptone Broth (SPB: 18 ml/vial) were purchased from Baltimore Biological Laboratories, Division of Becton, Dickinson & Co., Cockeysville, MD. Conversion of steroids by mixed faecal flora was examined in BHIC: dehydrated Brain Heart Infusion Broth (Baltimore Biological Laboratories) 37 g; cys-

tein HCl, 0.5 g; $NaHCO_3$; 1 g; 4 ml 0.025% aqueous resazurin (J. T. Baker Chemical Co., Phillipsburg, N.J.) and distilled water to 1 l. The medium was distributed in 200 ml amounts in 500 ml Erlenmeyer flasks and sterilised at 121°C for 20 min. Conversion by obligate anaerobes was studied in BHIC biologically reduced by addition of 0.1 ml of a 24 h old culture of *Escherichia coli* (BHIC-Ec; 6).

Sources of microorganisms. (a) Culture no. 116, isolated from human faecal flora, is an obligate anaerobe taxonomically related to *Eubacterium lentum* [6]; (b) *E. lentum*, neotype strain (VPI no. 0225) was kindly supplied by Drs. L. V. Holdeman and W. E. C. Moore, Virginia Polytechnic Institute, Blacksburg, Virginia; (c) *Clostridium paraputrificum* was recovered from human faecal flora [9]; (d) *Escherichia coli* was isolated from human faecal flora [6]; (e) Faecal samples from healthy adults on a Western diet were collected in stool cups under aerobic conditions, transported to the laboratory, and processed within 30 min of defaecation.

Pure cultures. The organisms were maintained in lyophilised form; prior to use, they were passaged 2–3 times in appropriate liquid media at 37°C; a 24 h culture was employed for conversion experiments.

Faecal suspension. Faecal specimens were decimally diluted to 10^{-3} in SPB.

Conversion experiments. Prior to incubation, aldosterone was added to the medium to give a concentration of 20 μg steroid/ml and 0.5% methanol (v/v), regardless of the media or the bacteria used for conversion.

The media were seeded with young cultures of obligate anaerobes or the dilute faecal suspensions (10^{-3}) in the proportion 20:0.1. The cultures were incubated at 37°C for 7 days.

pH and Eh. Using a Beckman Zeromatic II, these parameters were measured at the end of the incubation. Eh measurements were done with a platinum electrode. The instrument was checked regularly with quinhydrone-saturated buffers at pH 4 and 7. The readings were invariably within 10 mV of the theoretical values.

Extraction. The steroids were extracted as previously described [10].

Preparation of derivatives. Steroids extracts were oximated with 0.1 ml methoxyamine hydrochloride (2% in pyridine) for two days at room temperature and this was followed by 8 h silylation with 0.1 ml hexamethyldisilazane: trimethylchlorosilane (4:1). The derivatives were dried under nitrogen, sonicated with cyclohexane, centrifuged and transferred to clean vials. Under the conditions employed the C-18 carbonyl was incompletely converted to an oxime (Fig. 2) and a longer reaction period would be required for complete transformation [11].

Gas chromatography and GC/MS. Gas chromatography was initially carried out on a 20 m OV-101 capillary column in a Packard-Becker 409 according to methods previously described [12] and concentration and retention data of components were determined. The samples were then analysed by GC/MS using a Varian MAT 731 instrument with gas chromatograph housing a 2 m OV-1 packed column. The

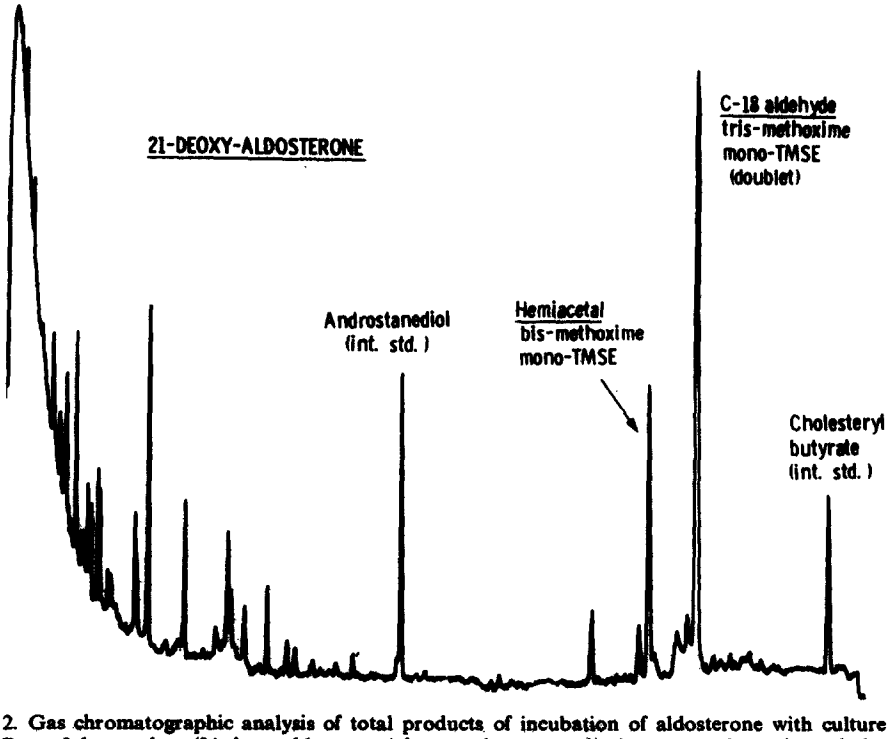

Fig. 2. Gas chromatographic analysis of total products of incubation of aldosterone with culture no. 116. Part of the product (21-deoxyaldosterone) has not been completely converted to tris-methyloxime and is still in hemi-acetal form. The results prove that more than 90% conversion has taken place and a single major product was obtained.

instrument was operated in continuous scanning mode (50–800 amu), data was stored on tape in mass converted form and required spectra were plotted.

RESULTS

Incubation with C. paraputrificum

In PR (Eh, −120 mV) and in BHIC-Ec(Eh, −300 mV), *C. paraputrificum* quantitatively reduced the 3-oxo-4-ene structure. The product had identical retention time to tetrahydraldosterone when analysed by gas chromatography and the mass spectrum was indistinguishable from that previously published for this steroid [11]. Control experiments revealed that *E. coli* was without effect on aldosterone.

Incubation with culture no. 116 and E. lentum

In BHIC-Ec (Eh, −300 mV), culture no. 116 quantitatively 21-dehydroxylated aldosterone. Only partial conversion was obtained at the lower Eh in PR. *E. lentum* also 21-dehydroxylated aldosterone in both media, albeit less completely.

Gas chromatographic/mass spectrometric analysis of the products revealed that under the optimum conditions only 21-deoxyaldosterone was found (Fig. 2). The mass spectrum of the trimethyloxime-mono TMS derivative is illustrated in Fig. 3. The molecular ion was at *m/e* 503 and prominent ions were formed by loss of methoxyl group (M-31) and trimethyl-silanol (M-90, M-90-31). Significant fragments are also seen at *m/e* 456, 457, (M-46, 47) and 440, 441 (M-62, 63), ions characteristic of aldosterone and metabolites with oximated C-18 carbonyl.

Incubation with a mixture of C. paraputrificum *and culture no. 116*

The experiments were performed in BHIC-Ec, since this medium has given the most complete conversions in the investigations described above. As could be expected, the joint effect of the two organisms was to metabolise aldosterone to its 21-dehydroxylated tetrahydro-derivative. 21-Deoxytetrahydroaldosterone was essentially pure and the mass spectrum of the bismethyloxime-bistrimethyl-silyl ether is illustrated in Fig. 3. The molecular ion was *m/e* 550 and prominent ions were at *m/e* 519 (M-31), 503 and 504 (M-46, 47) 487 and 488 (M-62, 63), 429 (M-90-31) and 339 (M-90-90-31).

Incubation with faecal flora

In BHIC seeded with the dilute faecal suspension aldosterone was converted to a single product. This product had identical gas chromatographic and mass spectrometric properties to 21-deoxy-tetrahydroaldosterone identified above.

DISCUSSION

Our interest in steroid C-21 reduction stems in part from studies of patients with 17α-hydroxylase deficiency syndrome, a disorder resulting in hypersecretion of corticosterone. Approximately 16–50% of the corticosterone secreted is excreted in the urine as 21-deoxy compounds formed by microbial reduction in the gut. These reduced metabolites are reabsorbed prior to urinary excretion [13]. The fraction of steroid excreted in 21-dehydroxylated form reflects the extensive biliary excretion of corticosterone rather than the

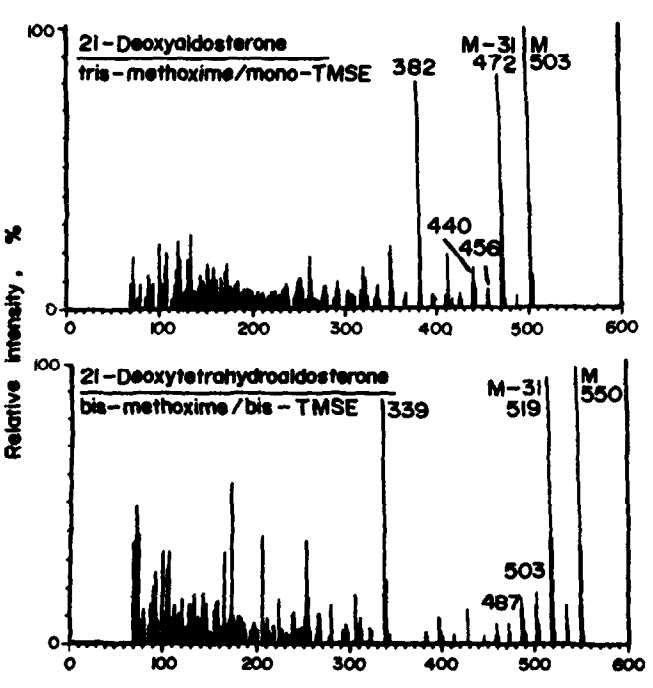

Fig. 3. Mass spectra of derivatives of 21-deoxyaldosterone and 21-deoxytetrahydroaldosterone.

particular susceptibility of this steroid to microbial metabolism. Cortisol, for example, is also readily 21-dehydroxylated by these organisms (unpublished observation) but is only excreted to a limited extent (4%) in bile and its 21-deoxy metabolites are not prominent urinary steroids [14]. Accurate data on the excretion of aldosterone in bile are not available [15] so the amount reaching the intestine is unknown. The detection of 21-deoxy metabolites of aldosterone by Kelly and co-workers in urine [1] suggested biliary excretion of aldosterone metabolites. The assumption was based solely on the recent knowledge that C-21 reduction cannot be carried out by mammalian enzyme systems, thus implicating the intestinal flora. Our present experiments indicate that mixed faecal flora transforms aldosterone to a single product [21-deoxytetrahydro-aldosterone (Fig. 1, I)] which can be accounted for by the joint action of *C. paraputrificum* and culture no. 116 or *Eubacterium lentum*. This metabolite was one of those identified by Kelly and colleagues [1].

There appears to be relatively limited structural requirements for reduction by *Eubacterium lentum* or culture no. 116 since the following steroids with an α-ketol side chain have proved susceptible to transformation; DOC, corticosterone, Compound A, cortisone, cortisol and 6β-hydroxy DOC. Reduction of the C-20 carbonyl, however, effectively blocks the C-21 dehydroxylation [5, 9] and so in the important 21-deoxy-20-dihydro- metabolites of corticosterone the reduction of the 20 carbonyl antecedes the reduction at C-21.

It is known that 80–90% of aldosterone is excreted in the urine so metabolites formed by intestinal bacteria following biliary excretion are almost entirely reabsorbed. With corticosterone the amount of 21-deoxymetabolites excreted in urine approximates the amount excreted in bile indicating almost total reduction during enterohepatic passage. If the same is true for aldosterone then biliary excretion would account for approximately 10% since this is the proportion of aldosterone metabolites identified in urine which do not contain a C-21 hydroxyl, i.e. 3α-hydroxypregnan-(11β-18), (18-20)-dioxide and 21-deoxytetrahydroaldosterone (Fig. 1). The bicyclic acetal was found to predominate in urine (accounting for 8% of metabolised aldosterone) and Kelly and Liebermann[1] suggest that it is formed by reduction of the C-20 carbonyl to C-20α hydroxyl which in turn condenses with the hydroxyl of the C-18 hemiacetal. Since the bicyclic actal was not identified following incubation of aldosterone with faecal flora it must be formed by liver or renal metabolism following intestinal reabsorption of the 21-deoxymetabolites. Now that these 21-deoxymetabolites of aldosterone have been synthesized *in vitro* our familiarity with their retention times and mass spectra will enable us to seek the endogenous metabolites in urine from patients with hyperaldosteronism.

The success of the study was due to the extreme selectivity of the bacterial reactions. Under the conditions used *C. paraputrificum*, *E. lentum* and Culture no. 116 effectively only produced single metabolites from a given substrate and the yields were invariably virtually quantitative. We therefore propose that these organisms be used for the small scale production of rare reference steroids which are unavailable commercially or available at prohibitive cost. Microbial reductions would offer a reliable and inexpensive approach to the problem. However, it should be pointed out that not all species of *C. paraputrificum* and *E. lentum* synthesise the required enzymes.

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