Inhibition of Human Testicular Glucose-6-Phosphate Dehydrogenase by Unsaturated C_{19}-Steroids

In previous publications[1,2], the inhibition of human red blood cell or placental glucose-6-phosphate dehydrogenase (G-6-PDH) by various C_{19}- and C_{21}-steroids has been reported. From these investigations a definite relationship could be established between the chemical structure of the particular steroid and its inhibitory activity. In continuation of such experiments the effect of 3β-substituted Δ^4- or Δ^5-C_{19}-steroids upon human testicular G-6-PDH was studied.

Testicular tissue was obtained from a 69-year-old male patient undergoing orchidectomy due to cancer of the prostate. After homogenization in 5 ml 0.9% sodium chloride/0.025% EDTA per g of wet tissue and a 20 min centrifugation at 18,000 *g*, the supernatant was submitted to an ammonium sulfate precipitation at 35% saturation. The precipitate was dissolved in 10 ml 0.05 *M* triethanolamine/0.005 *M* EDTA buffer of pH 7.6 and treated with 140 ml of a 13.1% suspension of hydroxyl apatite in 0.001 *M* phosphate buffer of pH 6–8 (SERVA Feinbiochemica, Heidelberg). Following a centrifugation the adsorbed enzyme activity was eluted from the precipitate by means of 200 ml 0.1 *M* phosphate buffer of pH 7.6. To the eluate sufficient ammonium sulfate was added to give a 50% saturation. Subsequently, the suspension was centrifuged for 20 min at 18,000 *g* and the precipitate dissolved in 6.0 ml triethanolamine/EDTA buffer. All assays of G-6-PDH activity were performed at 25 °C in 0.1 ml of the purified enzyme preparation, 3.0 ml triethanolamine/EDTA buffer, 0.1 ml 0.03 *M* NADP, 0.02 ml dioxan, eventually containing the steroid and 0.05 ml glucose-6-phosphate solution of varying concentration. The final concentration of steroid corresponded to a $10^{-5}M$ solution. From changes in the absorbance at 366 nm, registerd over 10 min, the enzyme activity was estimated and the inhibition constant K_i determined by the method of HUNTER and DOWNS[3].

Whereas the crude enzyme preparation exhibited a specific activity of 2.4 mU/mg protein, the purified enzyme possessed a specific activity of 126 mU/mg protein, indicating a 53-fold purification. The K_M-value of the purified enzyme was found to be $9.8 \times 10^{-5}M$ for glucose-6-phosphate as substrate. The inhibition of the enzyme by different steroids is demonstrated by their K_i-values in the Table. As can be seen 3β-hydroxy-4-androsten-17-one turned out to be the most effective inhibitor of the various steroids tested. This steroid with its equatorial hydroxy group and a planar half-chair configuration of rings A and B even surpassed 3β-hydroxy-5-androsten-17-one (dehydroepian-drosterone). The reduction of the 17-oxo group in the latter steroid, yielding 5-androstene-3β, 17β-diol (androstenediol), led to an almost complete loss of inhibitory activity. Additional functional groups, such as hydroxy groups at C-7, C-16 or C-19, reduced the biological activity of dehydroepiandrosterone as the parent compound to a considerable degree. Likewise, the replacement of the 3β-hydroxy group by a 3β-chloro group resulted in a remarkable decrease of activity. At the same time it can be stated that the 17-oxo group is required for inhibitory activity of C_{19}-streoids, as evidenced by the rather high K_i-value of 3β, 17β-dihydroxy-5-androsten-16-one. In every respect such findings agree with former results[1] and support the assumption that the presence of a 17-oxo-group, an equatorial hydroxy group at C-3 in the planar ring A/B configuration of 5α-, Δ^4- or Δ^5-C_{19}-steroids, as well as the lack of additional functional groups near C-5 provide the optimal structure for steroid inhibitors of G-6-PDH in the human organism. Since under physiological conditions, however, the levels of the more active steroids hardly reach a $10^{-6}M$ concentration – except perhaps in placental tissue – the participation of free C_{19}-steroids in the regulation of human G-6-PDH activity seems unlikely.

Inhibition of human testicular glucose-6-phosphate dehydrogenase by 3β-substituted Δ^4- or Δ^5-C_{19}-steroids

Steroid	K_i-value (for G-6-P)
3β-hydroxy-4-androsten-17-one	$6.6 \times 10^{-6}M$
3β-hydroxy-5-androsten-17-one	7.6
3β,16α-dihydroxy-5-androsten-17-one	$1.3 \times 10^{-5}M$
3β-hydroxy-5-androstene-16,17-dione	1.7
3β,7β-dihydroxy-5-androsten-17-one	2.9
3β,7α-dihydroxy-5-androsten-17-one	4.4
3β-chloro-5-androsten-17-one	4.6
3β-hydroxy-5-androstene-7,17-dione	6.9
3β,19-dihydroxy-5-androsten-17-one	8.6
3β,17β-dihydroxy-5-androsten-16-one	$1.4 \times 10^{-4}M$
5-androstene-3β,17β-diol	2.8
5-androstene-3β,16α, 17β-triol	4.5

Zusammenfassung. Die Bebrütung einer gereinigten G-6-PDH aus menschlichem Testisgewebe mit 3β-substituierten Δ^4- oder Δ^5-C_{19}-Steroiden zeigte, dass 3β-Hydroxy-Δ^4- oder Δ^5-androsten-17-on auch wirksame Inhibitoren der testikulären G-6-PDH darstellen. Zur gleichen Zeit konnten die früher gefundenen Zusammenhänge zwischen chemischer Struktur eines Steroids und seiner biologischen Aktivität im G-6-PDH-Hemmtest uneingeschränkt bestätigt werden.

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Determination of Methylguanidine in Serum and Urine from Normal and Uremic Subjects

The activated charcoal employed in the method of YATZIDIS et al.[1] for measuring monosubstituted guanidines in serum, catalyzes the oxidation of creatinine (CR) and creatine to methylguanidine (MG)[2]. Our previous observations on the MG contents in serum and urine obtained with a modification of this procedure[3,4], are falsely high and we retract them.

In the present paper a method is described to measure MG in body fluids, and the serum concentrations, as well as the daily urinary outputs found with it in normal subjects and in uremic patients, are reported.

Such procedure is based on the use of a strong cation-exchange resin to separate MG from creatine and CR, from the other Sakaguchi-reacting materials (arginine, guani-

dinioacetic and guanidinosuccinic acid) and from ammonia, which interferes with the color formation. A high sensitivity (lower limit: 0.005 mg/100 ml) is obtained by performing the Sakaguchi reaction on a low volume of fluid containing all the MG eluted quantitatively from the resin.

Method. Dowex resin (50-W 12, 100–200 mesch) is converted to the Na form by running 50 ml of 0.1 *N* NaOH through the column (6 × 20 mm). 20 ml for normal serum, 10 ml for uremic serum and normal urine and 2–5 ml for urine from uremic patients are made up to 100 ml with 0.01 *N* NaOH and run through the column, followed by 100 ml of 0.1 *N* NaOH and by 50 ml of deionized water. After this is passed completely, 50 ml of 2 *N* HCl are run, collected in a 200 ml beaker, and dried at 70°–80 °C in a ventilated oven. The dry residue is dissolved in 10 ml of 0.1 *N* NaOH which are filtered through paper and the Sakaguchi reaction (according to YATZIDIS et al.[1]) is performed on 8 ml of the clear filtrate. Color is read at 500 nm against a blank consisting of 8 ml of 0.1 *N* NaOH containing 15 g/l of NaCl. The standard curve is made by treating 10 ml aliquots of dilutions from 0.005 to 0.500 mg/100 ml of MG (as base) exactly like biological fluids.

Results. The percent recoveries of MG added (in amounts from 0.5 to 50 μg) to 10 ml of serum and urine from normal and uremic subjects, ranged from 85 to 102 (mean 93). In 15 samples of normal human serum the average MG concentration was 0.008 ± 0.002 mg/100 ml and its daily urinary output in 8 healthy adults on a freee diet (2 measurements each in consecutive days) was 1.83 ± 0.29 mg (concentrations ranged from 0.07 to 0.20 mg/100 ml).

In 82 acute and chronic renal patients, serum concentrations of MG ranged from normal values in the slightest cases (plasma CR concentrations below 2 mg/100 ml), to a maximum value of 0.60 mg/100 ml in an anuric patient (polycistic kidney disease) with a plasma CR content of 26 mg/100 ml. A direct correlation was found between the serum levels of CR and those of MG, but the ratio: serum MG/serum CR was significantly higher in the anuric patients. The daily urinary excretion of MG was found to be elevated in patients with renal failure (68 determinations in 35 cases) reaching values as high as 30 mg/24 h in the most severe ones (concentrations raeched levels of 3.0 mg/100 ml).

Discussion. Paper chromatography and electrophoresis performed on the dry residue of the HCl eluate dissolved in ethanol, revealed the only Sakaguchi-positive spot of MG thus showing that the procedure described is specific for it.

The elution of MG from the resin column with 60 ml of 4 *N* KOH, according to Carr et al.[5], and the reading of the color developed in it on a suitable standard curve, yielded results not significantly different from those obtained with the procedure described, provided the MG concentrations in the body fluid samples were higher than 0.20 mg/100 ml. This alkaline elution is not an advantageous alternative to the acid one, for it reduces the sensitivity and the accuracy of the method; however, the equality of the results obtained with the two procedures permits us to exclude that unknown substances (other than creatine and CR which are quantitatively removed from the resin with the preliminary elution with 0.1 *N* NaOH) are converted to MG during drying of the HCl eluate.

It can thus be concluded that MG is present in normal serum and urine and that it is retained in humans suffering for renal failure, as it was previously found to occur in nephrectomized dogs[5]. The reason for which high amounts of MG are excreted by uremics, as previously found by STEIN[6], is unclear at present.

Normal dogs severely intoxicated with MG suffer from symptoms resembling those of uremia[7], and our unpublished observations demonstrate that equal though milder symptoms appear even when their plasma MG levels are maintained as high as those found in severely uremic patients[8].

Riassunto. Viene descritto un metodo di dosaggio della metilguanidina nel siero e nell'urina e vengono riportati i valori normali della concentrazione serica (0.008 ± 0.002 mg/100 ml) e della eliminazione urinaria (1.83 ± 0.29 mg/24 h). Tanto la concentrazione serica quanto la eliminazione urinaria degli uremici sono risultate elevate.

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The Incorporation of 4-^{14}C-Cholesterol into Different Cholesterol Esters of the Blood Serum in Man, Guinea-Pig, Rat and Rabbit

Factors influencing the level of cholesterol esters in blood are at present not fully defined. Nevertheless there is increasing evidence about the importance of the esterification of cholesterol in the blood plasma per se (see the comprehensive survey by GOODMAN[1]).

At present it is generally accepted that the enzyme responsible for the esterification of cholesterol in the plasma of man, rat and rabbit is lecithin-cholesterol acyltransferase[2–4]. There appears to be some uncertainty whether the pattern of newly formed labelled cholesterol esters obtained after the incubation of a given plasma with radioactive cholesterol will resemble that existing normally in that plasma[4]. Therefore the experiments were carried out to investigate the cholesterol esterification in the serum of four species with different pattern of the cholesterol esters.

Fresh serum, obtained after centrifugation of blood which was allowed to clot at room temperature, was always used. 4-^{14}C-cholesterol (Radiochemical Centre, Amersham) adsorbed to celite has been added to the