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GLUCURONIDE FORMATION IN THE TRANSPORT OF TESTOSTERONE AND ANDROSTENEDIONE BY RAT INTESTINE

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SUMMARY

Incubation of [4-^{14}C]testosterone or Δ^4-androstene-3,17-dione with everted sacs of rat intestine resulted in the net transport of radioactivity to the serosal media. At the end of a 3-h incubation, both the mucosal and serosal media were found to contain radioactivity in compounds with the chromatographic properties of testosterone, Δ^4-androstene-3,17-dione, androstane-3,17-dione, androsterone, androstane-3α,17β-diol. Androstane-3α,17β-diol was primarily present in the serosal media as a glucuronide conjugate. This steroid was the only one thus conjugated. Accumulation of this glucuronide conjugate of androstane-3α,17β-diol in the serosal media was responsible for the net transfer of radioactivity across the gut wall.

INTRODUCTION

Glucuronide conjugation has been shown to be involved in the active transfer of certain thyroxine analogues from the mucosal to the serosal surface of everted sacs of rat intestine *in vitro*[1,2]. The intestinal wall is relatively impermeable to these glucuronide conjugates and this impermeability results in their accumulation in the serosal medium.

To determine whether this mechanism for transport might have more general application, the ability of everted gut sacs to transport a number of androgens and estrogens has been examined. Using [^{14}C]estradiol-17β, it was observed that net transport of radioactivity to the serosal medium was accomplished by a two step process: the estradiol was first oxidized to estrone, and the estrone was conjugated as a glucuronide[3]. The accumulation of this conjugate in the serosal fluid resulted in levels of radioactivity as much as seven times greater than those in the mucosal medium.

In the present study, the ability of everted gut sacs to transport certain androgens has been examined. When either [4-^{14}C]testosterone or Δ^4-androstene-3,17-dione is incubated with everted sacs of small or large intestine, net accumulation of radioactivity in the serosal medium is observed. Both these compounds undergo a series of metabolic transformations before appearing in the serosal medium, chiefly as a glucuronide conjugate of androstane-3α,17β-diol.

EXPERIMENTAL

[4-^{14}C]Testosterone (specific activity 20 μC/mg) and Δ^4-androstene-3,17-dione (specific activity 7.8 μC/mg) were obtained from New England Nuclear and Picker X-ray Corporations. No radioactive impurities were detected when the compounds were chromatographed and scanned in a standard gas-flow strip scanner. Unlabelled standard steroids were obtained commercially or as generous gifts from Drs. L. AXELROD, S. LIEBERMAN or H. SCHEDL.

Wistar strain male rats weighing 150–250 g were decapitated and the intestine removed and rinsed in modified Ringer's solution. The intestine was everted and tied into weighed sacs 2–5 cm in length according to the method of WILSON AND WISEMAN[4]. Sacs were prepared using the lower half of the small intestine unless otherwise noted. Each sac was filled with 0.5 ml standard medium containing 0.134 M NaCl, 0.011 M KCl, 0.008 M phosphate buffer (pH 7.4), 0.0001 M $CaCl_2$ and 0.02 M glucose. The sac was then placed in a 20-ml beaker containing 4.0 ml of the same medium plus [4-^{14}C]testosterone ($3.5–5.8 \cdot 10^{-6}$ M) or [4-^{14}C]Δ^4-androstene-3,17-dione ($1.1–2.2 \cdot 10^{-5}$ M).

The sacs were incubated for 120–180 min at 37° in a Dubnoff shaker oscillating 120 times/min, with oxygen flowing at 4–5 ft^3/h. At the end of the incubation, 0.2-ml aliquots of the serosal and 0.5-ml aliquots of the mucosal medium were counted in aluminum planchets with a Geiger–Müller tube; sample counts generally exceeded 10 times background. Transport was calculated as the ratio of counts/min/unit volume serosal to mucosal medium. Chromatography of the mucosal and serosal media was performed on Whatman No. 1 paper using BUSH A, B_1, B_3, or B_5 solvent systems[5]. Radioactivity on the strips was located with a gas-flow-strip scanner.

In experiments where large amounts of the unknown transformation products were isolated for identification, sacs 5–6 cm in length were filled with 1.0 ml of standard medium and placed in beakers with 8 ml of medium containing either $2.8 \cdot 10^{-4}$ M [4-^{14}C]testosterone or $2.2 \cdot 10^{-5}$ M [4-^{14}C]Δ^4-androstene-3,17-dione. The post-incubation serosal media were then combined and placed on Whatman No. 3 paper. Following chromatography, each band of radioactivity was located and the steroid eluted for subsequent purification and identification. The steroid which was conjugated as a glucuronide remained at the origin when solvent was allowed to pass through the area for 20–30 h. Following this procedure the radioactivity could be easily extracted from the paper with distilled water. The conjugate so prepared was hydrolyzed with "Ketodase" (1000 units/ml) during a 6- or 27-h incubation in 0.5 M acetate buffer (pH 5) at 37°. The steroid thus released was then purified by paper and column chromatography for further identification.

Tentative identification of these isolated unknown compounds was provided by running the unknowns and reference standards on parallel strips in the same tank at the same time in several different BUSH solvent systems. Unlabelled compounds were located with Zimmerman and phosphomolybdic acid reagents and with ultraviolet light. In addition, the unknown steroids were chromatographed with various known reference standards applied to the same strip.

Partition chromatography of several of the unknown steroids was performed using a Celite column developed with a benzene–cyclohexane–propyleneglycol–methanol (1:1:1:1, v/v) system modified from Kochakian[5]. Radioactivity in aliquots of

each fraction was measured and the rest of each fraction used to assay for the unlabelled standards. This was accomplished by taking the fraction to dryness, adding 10% phosphomolybdate in methanol and heating in a water bath of 80–90° until the methanol evaporated. The dried aliquots were redissolved in methanol and the absorbancy read at 610 mμ.

Gas chromatography of the steroid released by hydrolysis of the conjugate was performed using a Barber-Colman model 10 Gas Chromatography apparatus. A column of 2% SE-30 on chromabsorb W (30–60 mesh) was used with a gas flow of 100 ml/min of argon. The analysis was performed at a temperature of 205°. Analysis of a known reference sample of androstane-3α,17β-diol with a quantity–response determination of this compound was carried out, followed by analysis of the unknown.

Two types of experiments were performed to test the possible action of bacteria in these studies. In the first, an everted segment of gut 14 cm in length was filled with 5 ml of unlabelled medium and incubated in 5 ml of the same medium for 1 h at 25°. The inner and outer fluids were combined and incubated for 3 h at 37° with 100 μg of labelled testosterone. Following this, the medium was examined in the usual manner for transformation products. The second type of study was performed with sterilized gut-sac preparations. Six rats were placed on a diet of 10 g of Rockland D-free rat mash with Wesson oil for four days. To the diet of four rats were added 200 mg tetracycline, 2500 units bacitracin, and 25 mg neomycin sulfate per day per rat. Stool cultures were taken before the antibiotics were begun and on the third and fourth day of the regimen. The animals were than sacrificed and the usual studies performed. Six intestinal sacs were prepared from every animal, two each from the upper, mid and lower portions of the small intestine.

RESULTS

When everted gut sacs were incubated in standard medium containing labelled testosterone or androstenedione, radioactivity accumulated in the serosal media in concentrations as much as eight times greater than those in the mucosal media. As can be seen in Table I, the ratio established was to some extent dependent upon both the concentration of steroid and upon the portion of the gut used. For example, with testosterone the mean ratio was 2.4 at the lower concentration of steroid and either 1.2 or 1.6 at the higher concentration, depending upon whether sacs from the upper or lower half of the small intestine were used. When androstenedione was the incubated steroid, the mean ratio was 1.8. The entire intestine, from duodenum to colon, possessed the capacity to accumulate radioactive material in the serosal

TABLE I

ACTIVE TRANSFER OF ANDROGENS FROM MUCOSAL TO SEROSAL MEDIA

Steroid	*Portion of intestine*	*No. of sacs*	*Mean ratios (serosal/mucosal media)*
Testosterone ($3.5–5.8 \cdot 10^{-6}$ M)	Entire gut	88	2.4
Testosterone ($2.8 \cdot 10^{-4}$ M)	Upper half small gut	108	1.2
Testosterone ($2.8 \cdot 10^{-4}$ M)	Lower half small gut	95	1.6
Δ^4-Androstene-3,17-dione ($1.0–2.2 \cdot 10^{-5}$ M)	Entire gut	14	1.8

medium; the mean ratios were consistently highest in the lower portion of the small intestine and in the colon.

Examination of the post-incubation serosal media of gut sacs from the lower half of the small intestine revealed that the major peak (50–90 %) of radioactivity was in the form of a water-soluble conjugate (conjugated Unknown I) which remained at the origin in all developing systems used (BUSH A, B_1, B_3, and B_5). In those sacs where accumulation of radioactivity in the serosal medium was greatest, the largest amounts of conjugate were observed; conversely, in sacs with lower gradients, less

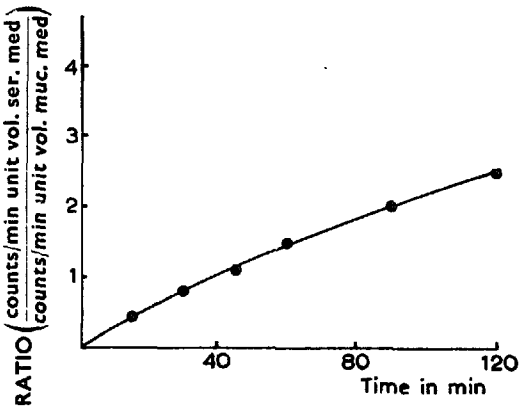

Fig. 1. Effect of time on transport of [4-^{14}C]testosterone by everted gut sacs. The results plotted are the average of two separate time curves using two different rats.

conjugate was consistently found. In the upper half of the small intestine, an as yet unidentified compound (Unknown VI) was demonstrated in quantities which were frequently sufficient to give the major peak of radioactivity in the post-incubation serosal medium. In addition to the conjugated steroid and the unidentified compound (Unknown VI), five other unconjugated steroids (Unknown I–V) were detected in the serosal fiuid and their identities defined by paper chromatography. The same six metabolic products were found whether androstenedione or testosterone was the incubated steroid. The same products were also formed when gut sacs from female rats were used.

In the post-incubation mucosal medium, on the other hand, very little of the radioactivity was present as the vater-soluble conjugate. From 70–90 % of the radioactivity migrated with R_F values compatible with the unconjugated steroids (Unknown I–VI).

The addition of various metabolic inhibitors decreased the transport of radio-

TAPLE II

EFFECT OF VARIOUS METABOLIC INHIBITORS ON TRANSPORT WITH $3.4–5.8 \cdot 10^{-6}$ M [4-^{14}C]TESTOSTERONE

Inhibitor	*No. of animals*	*No. of sacs*	*Mean ratio (serosal/mucosal)*
None	14	26	1.52
Sodium fluoride (0.002 M)	2	4	1.02
Sodium cyanide (0.0005 M)	4	7	0.99
Sodium azide (0.003 M)	4	8	0.99
2,4-Dinitrophenol (0.0005 M)	4	8	1.00

TABLE III

RELATIVE QUANTITIES OF EACH COMPOUND PRESENT IN THE SEROSAL AND MUCOSAL MEDIA AT VARYING TIME INTERVALS

Determination by chromatography of each medium in BUSH B_1, which does not separate Unknowns I and II, or Unknowns III, IV, V.

	Conjugate	*Unknowns I and II (androstane-3α-17β-diol, testosterone)*	*Unknowns III, IV and V (Δ^4-androstene-3,17-dione, androstane 3,17-dione, androsterone)*	*Unknown VI*
Serosal				
0 min	−	−	−	−
15 min	±	±	±	−
30 min	+	+	+	±
45 min	++	+	+	+
60 min	++	±	++	±
90 min	+++	±	++	±
120 min	++++	±	++	±
Mucosal				
0 min	−	++++	−	−
15 min	±	++++	±	±
30 min	±	+++	+	±
45 min	±	++	++	±
60 min	+	+	++	+
90 min	+	+	++	+
120 min	++	±	++	+

activity into the serosal media and prevented the development of a concentration gradient (Table II). These inhibitors markedly diminished or completely prevented the production of the water soluble steroid conjugate as well as of the unidentified compound (Unknown VI). Although each of the other steroid transformation products (Unknowns I–V) could still be detected, their production was also significantly depressed in the presence of the various inhibitors.

When consecutive gut sacs were incubated for varying intervals of time in a medium containing [^{14}C]testosterone, the ratio of radioactivity in the serosal medium to that in the mucosal medium increased with time (Fig. 1). Unity was exceeded after 45–60 min of incubation, depending upon the portion of intestine used to prepare the sacs; the shorter period of time was required with sacs from the lower part of the small intestine. Chromatography of the serosal and mucosal media was performed at each time interval. As can be seen in Table III, the quantity of unchanged testosterone in the outside media gradually decreased with time. The decrease coincided with the accumulation of conjugated steroid in the inside medium.

Properties of the conjugate

The steroid conjugate was water-soluble and could be easily eluted from the chromatography paper with small amounts of distilled water. The eluate, when rechromatographed, gave a single band of radioactivity which remained at the origin in all the developing systems used (BUSH A, B_1, B_3, and B_5). The conjugate was

stable when incubated in distilled water (pH 6) for 27 h at either 25° or 37° as well as when incubated in 2 N NaOH for 24 h at 37°. The conjugate could be hydrolyzed, however, by boiling for 10 min in 2 N HCl or by incubation with β-glucuronidase (EC 3.2.1.31) for 6 h at 37°. The cleavage of the conjugate with β-glucuronidase was completely inhibited by the addition of 0.001 M 1,4-saccharolactone, a specific inhibitor of β-glucuronidase[6]. On the basis of these findings, it is assumed that the conjugate is a glucuronide.

When the glucuronide conjugate was itself incubated with everted sacs of the lower jejunum, it was not actively transferred into the serosal medium. In four animals, the ratios of radioactivity in the serosal media to those in the mucosal media ranged from 0.21–0.23 after 1 h and from 0.22–0.77 after 3 h of incubation. Paper chromatography of the media inside and outside the gut sacs after 3 h incubation with high concentrations of the glucuronide conjugate revealed that approx. 10% of the conjugate had broken down. Small amounts of each of the Unknowns (I–VI) could be detected.

Properties of the conjugated steroid

When the glucuronide isolated from the serosal media was hydrolyzed, all of the radioactivity was released in a single unconjugated steroid (Unknown I). The

TABLE IV

CONJUGATED STEROID (UNKNOWN I) AFTER HYDROLYSIS WITH β-GLUCURONIDASE

	R_F Values (Bush)			
	A	B_1	B_3	B_5
Unknown	0.03–0.09	0.64–0.72	0.50–0.59	0.80–0.89
Androstane-3α,17β-diol	0.00–0.10	0.64–0.72	0.48–0.55	0.80–0.89

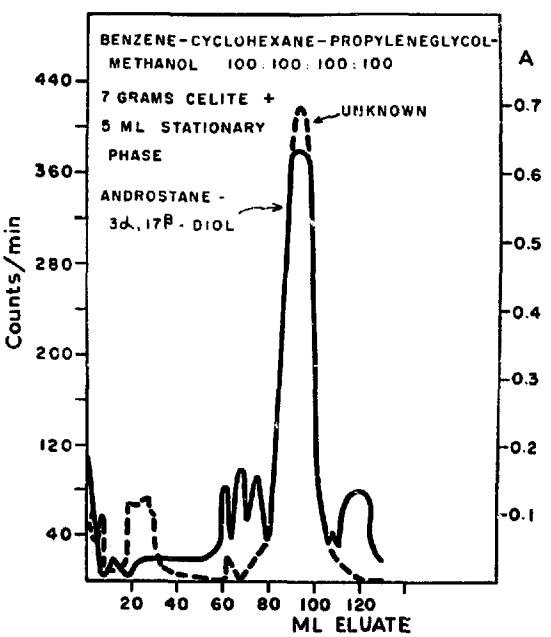

Fig. 2. Simultaneous partition chromatography of the unknown conjugated steroid with androstane-3α,17β-diol.

mobilities of this steroid were compared with those of known unlabelled steroids which were run concomitantly in the same tank on parallel strips. It had R_F values identical to those of known androstane-3α,17β-diol in BUSH systems A, B_1, B_3, and B_5. Further, it did not separate from known androstane-3α,17β-diol when applied to the same strip (Table IV). The unknown was not visible in ultraviolet light when present in quantities greater than 25 μg and gave a negative Zimmermann and a positive phosphomolybdate reaction.

TABLE V

KNOWN STEROIDS DEVELOPED IN BUSH SYSTEMS A, B_1, B_3 AND B_5 FOR IDENTIFICATION OF UNKNOWNS

	I. Dioxygen steroids
(*A*)	*Androstene series*
1	Δ^5-Androstene-3β-ol,17-one
2	Δ^4-Androstene-3,17-dione
3	Δ^4-Androstene-17α-ol,3-one
4	Δ^4-Androstene-17β-ol,3-one
(*B*)	*Androstane series*
5	Androstane-3,17-dione
6	Androstane-3α-ol,17-one
7	Androstane-3β-ol,17-one
8	Androstane-17β-ol,3-one
9	Androstane-3α,17β-diol
10	Androstane-3β,17α-diol
11	Androstane-3β,17β-diol
(*C*)	*Etiocholane*
12	Etiocholane-3α-ol,17-one
13	Etiocholane-3α,17β-diol
	II. Trioxygen steroids
(*A*)	*Androstene series*
14	Δ^5-Androstene-1α,3β-diol,17-one
15	Δ^5-Androstene-3β,16α,17β-triol
16	Δ^4-Androstene-3,11,17-trione
17	Δ^4-Androstene-6β-ol,3,17-dione
18	Δ^4-Androstene-11α-ol,3,17-dione
19	Δ^4-Androstene-11β-ol,3,17-dione
20	Δ^4-Androstene-17β-ol,3,11-dione
21	Δ^4-Androstene-2β,17β-diol,3-one
22	Δ^4-Androstene-11α,17β-diol,3-one
23	Δ^4-Androstene-11β,17β-diol,3-one
(*B*)	*Androstane series*
24	Androstane-3,11,17-trione
25	Androstane-11β-ol,3,17-dione
26	Androstane-3α,11β-diol,17-one
(*C*)	*Etiocholane series*
27	Etiocholane-3,11,17-trione
28	Etiocholane-3α-ol,11,17-dione
29	Etiocholane-3α,11β-diol,17-one
30	Etiocholane-3α,17β-diol,11-one
	III. Estrogens
31	Estrone
32	Estradiol

By partition chromatography using a Celite column developed with a benzene–cyclohexane–propyleneglycol–methanol system, Unknown I and standard androstane-3α,17β-diol gave identical elution patterns when applied simultaneously (Fig. 2). Finally, in analysis by gas chromatography kindly performed by Dr. B. KLIMAN of the National Institutes of Health, the unknown compound appeared after the same time interval as did the known androstane-3α,17β-diol when run at the same temperature and flow rate during one experimental period.

Identification of the steroid transformation products

Incubation of either testosterone or Δ^4-androstene-3,17-dione with everted gut sacs yielded the same transformation products. Tentative identification of the unconjugated metabolites (Unknowns II–V) was obtained by paper chromatography after isolating each compound from the ncubation media by paper chromatography and purification by development in a variety of chromatography systems. Each of the known steroids listed in Table V was run on parallel strips with each of the unknowns in the various BUSH systems. In addition, the standard steroids were mixed with each of the unknowns and the mixtures chromatographed and developed on the same strips. The transformation products were tentatively identified by these methods as testosterone (Unknown II), Δ^4-androstene-3,17-dione (Unknown III), androstane-3,17-dione (Unknown IV), and androsterone (Unknown V). Small amounts of un-

TABLE VI

CHROMATOGRAPHIC IDENTIFICATION OF TRANSFORMATION PRODUCTS (UNKNOWNS II–V)

	R_F Values (BUSH)			
	A	B_1	B_3*	B_5
Unknown II	0.04–0.09	0.70–0.76	0.53–0.54	0.87
Testosterone	0.03–0.10	0.70–0.76	0.54	0.87
Unknown III	0.24–0.30	0.81–0.90	0.76–0.83	0.87–0.89
Δ^4-Androstene-3,17-dione	0.24–0.29	0.81–0.90	0.76	0.88
Unknown IV	0.51–0.57	0.81–0.90	0.83–0.87	0.89–0.95
Androstane-3,17-dione	0.51–0.59	0.83–0.91	0.83	0.93
Unknown V	0.30–0.38	0.81–0.90	0.74–0.76	0.83–0.89
Androsterone	0.28–0.38	0.85–0.88	0.76	0.90

* The R_F's of standard steroids in this system proved to be extremely variable from day to day, presumably secondary to changes in room temperature and atmospheric pressure.

TABLE VII

COMPARISON OF UNKNOWN VI WITH VARIOUS REFERENCE STANDARDS

	R_F Values (BUSH)			
	A	B_1	B_3	B_5
Unknown VI	0.00–0.03	0.42–0.51	0.19–0.28	0.73–0.80
Androstane-3α,17β-diol,17-one	—	0.46–0.50	0.26–0.30	—
Androstane-3β,17β-diol	—	0.46–0.52	0.48–0.49	—
Etiocholane-3α,11β-diol,17-one	—	0.35–0.45	0.18–0.24	0.73–0.78
Etiocholane-3α,17β-diol,11-one	—	0.48–0.50	—	—
Etiocholane-3α,17β-diol	—	0.44	0.53	—
Estrone	—	0.51–0.53	0.65–0.73	0.90–0.91
Estradiol	—	0.32–0.40	0.31–0.35	0.73–0.74

conjugated androstane-3α,17β-diol (Unknown I) were also detected. The appropriate data are presented in Table VI.

One metabolic product (Unknown VI) remains unidentified. This compound was produced in large amounts by the upper half of the small intestine and its formation was prevented by non-specific inhibitors. Although its mobilities were similar to a number of the reference steroids, as can be seen in Table VII, it separated from each of these steroids in at least one of the BUSH systems employed. With androstane-3α,11β-diol-17-one, etiocholane-3α,11β-diol-17-one and etiocholane-3α,17β-diol-11-one, separation from Unknown VI was also achieved during partition chromatography using a Celite column developed with a benzene–cyclohexane–propyleneglycol–methanol system. This unidentified compound could not be hydrolyzed with 2 N HCl, 2 N NaOH, or with β-glucuronidase. It was not visible in ultraviolet light when present in quantities greater than 25 μg and gave positive Zimmermann and phosphomolybdate reactions. Several attempts to obtain an infrared spectrum of this compound were unsuccessful, either due to lack of sufficient purity even after several passages through both paper and column chromatographic systems or to some unusual configuration of the compound itself.

Studies utilizing sterilized gut

Two studies were carried out to eliminate the possibility that the steroid transformations observed might be due to the action of bacteria. When gut washings, prepared as described above, were incubated for 3 h at 37° with labelled steroids, no transformation products were detected. In a second experiment six rats were placed on a constant diet; tetracycline, bacitracin, and neomycin sulfate were added to the diet of four of them. The bacterial growth from the stools of all animals was heavy before the antibiotic administration and continued to be so in the case of the control rats. The stool cultures of the animals given antibiotics became negative by the third day. The transport, conjugation, and transformation products were identical when [^{14}C]testosterone was incubated with sacs from both groups of rats.

DISCUSSION

Metabolic transformations of both testosterone and Δ^4-androstene-3,17-dione were observed when these steroids were incubated with everted sacs of rat intestine. Such transformations have not previously been demonstrated using intestinal tissue. It is not surprising that they should occur, however, since similar metabolic products have been isolated following incubation of these steroids with other tissue such as liver and kidney. A possible pathway leading to the various transformation products which were identified is presented in Fig. 3.

Glucuronide conjugation in these preparations occurred with only one of the metabolites, androstane-3α,17β-diol. FISHMAN AND SIE[7] have reported the formation of testosterone glucuronide using rat-liver slices, but no evidence for such a conjugation was observed in this study. The accumulation of radioactivity in the serosal medium in concentrations significantly greater than in the mucosal medium was completely dependent upon the formation of this glucuronide conjugate of androstanediol.

The unidentified compound (Unknown VI) was formed in comparatively large quantities in the duodenal and upper jejunal regions of small intestine, and to a lesser

extent in the lower small intestine and colon. The evidence to date would suggest that this compound is a saturated steroid containing three oxygens, including at least one keto and one hydroxyl group. In 1956, AXELROD[8] described a number of tri-oxygen metabolites of testosterone, but none available for this study had chromatographic characteristics identical with those of the unknown. The formation of this unknown was prevented by concentrations of non-specific metabolic inhibitors which prevented glucuronide conjugation of androstanediol, but which only partially suppressed the formation of the other steroid intermediates. However, the unknown was completely resistant to procedures which should hydrolyze glucuronide or sulfate conjugates, and it seems unlikely that it is a simple conjugated steroid.

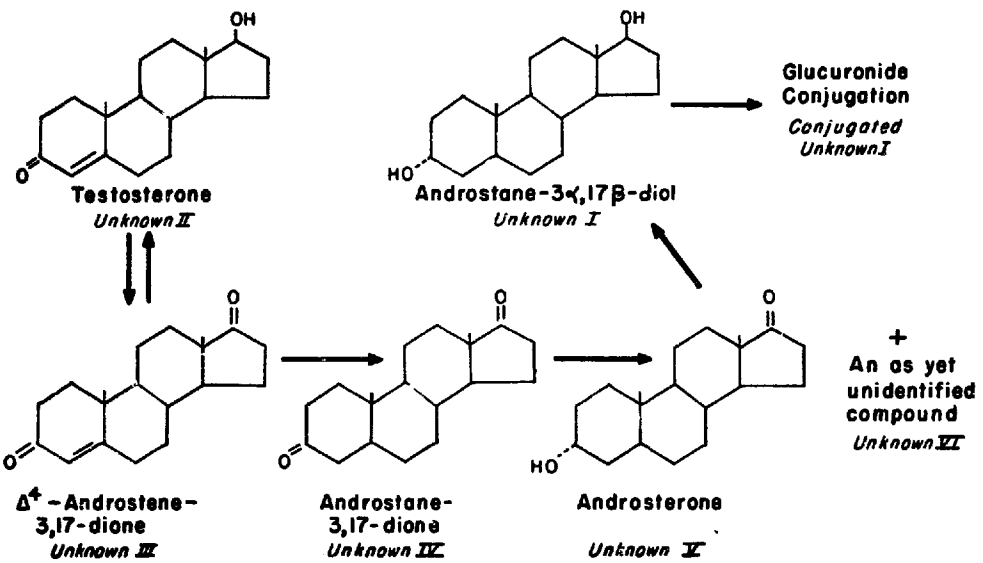

Fig. 3. A possible metabolic pathway responsible for the formation of the products isolated following incubation of either testosterone or androstenedione with everted gut sacs.

The relative inactivity of orally administered testosterone has generally been attributed to a rapid delivery of the steroid to the liver via the portal system. The results of the present investigation suggest the possibility that significant metabolism of orally administered testosterone may occur during passage through the intestinal wall.

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