and that the electron flow is reversed in light to regenerate the pool of reducing power with consequent oxidation of the NADPH.

The photoreduction of both NADP and cytochrome c has been shown by Keister *et al.* (1962) to require the presence of an enzyme, photosynthetic pyridine nucleotide reductase. The restoration of cytochrome c reduction by α-tocopherylquinone and by the PQC plus PQD mixture also requires addition of photosynthetic pyridine nucleotide reductase. The reduction of NADP restored by PQB, however, is different from the activity of the original chloroplasts since no requirement for photosynthetic pyridine nucleotide reductase can be shown (see Table IV). It is, of course, possible that a small amount of photosynthetic pyridine nucleotide reductase is present in the extracted chloroplasts and that this endogenous PPNR works with high efficiency when PQB is added. For the present, we must say that the restoration of NADP reduction by PQB does not resemble the original system in this lack of a requirement for photosynthetic pyridine nucleotide reductase. On the other hand, the photooxidation of reduced NADP which occurs when PQA is added to extracted chloroplasts is stimulated by addition of photosynthetic pyridine nucleotide reductase.

These results lead us to the proposal that there are several sites for quinone function in chloroplasts which show considerable specificity with regard to quinone type. Further experimentation will be necessary to determine the site of function of each of these quinones.

ACKNOWLEDGMENTS

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In Vivo Studies with Radioactive Steroid Conjugates. I. The Fate of Randomly Tritiated Androsterone Glucuronoside in Humans*

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Randomly tritiated androsterone glucuronoside was administered intravenously in trace amounts to three normal individuals. Urine was collected for 2–3 days and from these specimens pure androsterone glucuronoside was reisolated by chromatographic techniques. The distribution of the tritium between androsterone and glucuronic acid portions of the conjugate isolated from the urine was the same as that of the injected tracer. In one instance all the radioactivity present in the urine was found to be contained in androsterone glucuronoside. Furthermore in no instance was tritium found in the urinary androsterone sulfate. These results indicate that the androsterone glucuronoside present in urine is a metabolic end-product which, once formed, is neither cleaved *in vivo* nor further metabolized.

Metabolites of the steroid hormones as well as those of many other classes of compounds are found in the urine conjugated with either glucuronic acid or sulfuric acid. In the past, these "detoxified" products have been regarded merely as a means by which the excretion of waste products is facilitated. More recently the suggestion has been made (Fishman, 1961) that these conjugates may, in addition, serve as vehicles for the transport of biologically active compounds through the blood or perhaps through the walls of the target cells. The recent findings that dehydroisoandrosterone sulfate (Roberts *et al.*, 1961) and estrone sulfate (Twombley and Levitz, 1960) are freely interconvertible *in vivo* with their respective steroidal aglycones has caused a renewal of interest in this subject since it is evident that conjugated as well as free steroids must be taken into account before the metabolic pathways and the mode of action of these hormones can be fully understood. Further emphasis has been given to this facet of steroid metabolism by the recent demonstration that dehydroisoandrosterone sulfate is a normal secretory product of the adrenal gland in humans (VandeWiele *et al.*, 1963). That the adrenal gland can *in vivo* sulfate dehydroisoandrosterone (Wallace and Lieberman, 1963; Cohn *et al.*, 1962) as well as estrone (Sneddon and Marrian, 1963) has also been unequivocally demonstrated. Finally, the demonstration that pregnenolone sulfate may serve as a direct *in vivo* precursor of dehydroisoandrosterone sulfate without the cleavage of the sulfate ester group reveals a still undefined metabolic role for sulfate conjugates (Calvin *et al.*, 1963).

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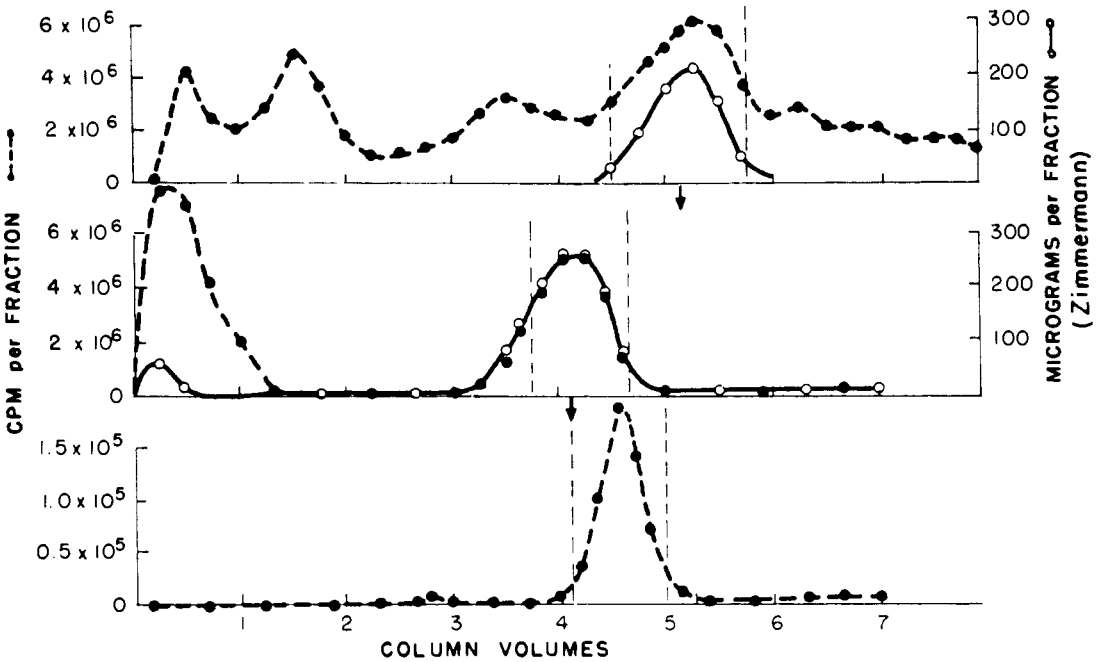

FIG. 1.—Purification of randomly labeled H^3-androsterone glucuronoside by column partition chromatography. Arrows indicate successive chromatographic procedures of fractions delineated by vertical broken lines; see text for details.

In general, the most abundant steroidal conjugates present in urine are those of glucuronic acid. Until now the only evidence that such glucuronosides may be further metabolized is a brief report (Kellie, 1961) which indicates that the oral ingestion of dehydroisoandrosterone glucuronoside by humans results in cleavage of the conjugate and further metabolism of the steroidal moiety to androsterone and etiocholanolone. The physiological significance of this experiment is unclear because this relatively minor (in quantitative terms) conjugate possessing a rather special structure (a 3β-glucuronosyl-Δ^5-unsaturated grouping) was administered by feeding and this unusual route of administration may have influenced the result.

In order to eliminate some of these uncertainties, the experiments reported in this paper were carried out to determine whether a more typical steroid glucuronoside (androsterone glucuronoside) can be cleaved *in vivo* to yield the free steroid. For this purpose, randomly tritiated androsterone glucuronoside was administered intravenously to three normal male subjects. Urine was collected for 2 or 3 days and from these urinary specimens the 17-ketosteroid conjugates were isolated. The following findings proved that cleavage of the injected glucuronoside did not occur. (1) The distribution of tritium between the steroid and the glucuronic acid portions of androsterone glucuronoside was the same for both the injected tracer and the conjugate recovered from the urine. Had cleavage occurred, the radioactive urinary conjugate would have had a larger proportion of its radioactivity in its steroid moiety since reconjugation could only have taken place with glucuronic acid of endogenous and therefore nonradioactive origin. (2) As determined by reverse isotope dilution procedures, all (107%) of the radioactivity in the urine from one experiment was found to be present as androsterone glucuronoside. (3) No evidence was obtained of the presence in the urine of significant amounts of other radioactive compounds, particularly androsterone sulfate.

EXPERIMENTAL

Androsterone glucuronoside (free acid) was generously made available to us by Dr. John Schneider who had isolated it from human urine. Chemical reagents and solvents were of analytical grade and were used as received unless otherwise stated in the text.

Steroid conjugates were extracted from urine with a mixture of ether and ethanol (Edwards *et al.*, 1953). Steroid sulfates were hydrolyzed by solvolysis (Burstein and Lieberman, 1958), and pure steroid glucuronosides were hydrolyzed by treatment with perchloric acid in anhydrous tetrahydrofuran at 50° for 3 hours (Jacobsohn and Lieberman, 1962). Urinary glucuronosides were hydrolyzed by incubation with β-glucuronidase at *p*H 5.0 and 37° for 3–4 days. Free steroids and their conjugates were chromatographed on Celite partition columns in the following solvent systems which have recently been reported (Siiteri *et al.*,1963).

System K-C (isooctane–*t*-butanol–water–concd NH_4OH, 200:500:475:25 [v/v/v/v]) was used for the group separation of 17-ketosteroid sulfates and glucuronosides.

System K-G (isooctane–*t*-butanol–water–glacial acetic acid, 300:500:500:125 [v/v/v/v]) was used for the separation of individual 17-ketosteroid glucuronosides.

Free 17-ketosteroids were chromatographed using system K-F (isooctane–*t*-butanol–water–methanol, 500:100:50:350 [v/v/v/v]). Free 17-ketosteroids were also purified by paper chromatography in the Bush A system (Bush, 1952).

Columns were prepared by the "dry pack" method previously described (Kelly *et al.*, 1962). The term *hold-back volume* used herein refers to the volume of mobile phase contained in a packed column, and elution volumes for a particular compound are expressed in these units.

17-Ketosteroids were determined by a modification (Callow *et al.*, 1938) of the Zimmermann reaction. Sulfate esters were measured as complexes with methylene blue (Crepy and Rulleau-Meslin, 1960). Radioactivity was determined with a Packard Tri-Carb liquid scintillation spectrometer. Dried samples of free steroids were assayed by solution in 5 ml of toluene containing 0.3% of 2,5-diphenyloxazole and 0.01% of 1,4-bis-2-(5-phenyloxazolyl)-benzene. Steroid

TABLE I

TRITIUM DISTRIBUTION IN TRACER ANDROSTERONE GLUCURONOSIDE

Mixtures of radioactive and nonradioactive androsterone glucuronoside were hydrolyzed with perchloric acid in experiments A and B, and with β-glucuronidase in experiment C. See text for details.

	Androsterone Glucuronoside		Androsterone	Tritium in Androsterone Moiety of Tracer (%)
Experiment	Tritium (cpm)	Weight of Androsterone (μg)	Specific Activity after Hydrolysis	
A	181,000	1120	121.1	74.9
B	354,000	1200	202.1	68.6
C	141,000	—	—	71.4
			Mean:	71.6

conjugates and crude samples were counted similarly except that the dried sample was first dissolved in 1 ml of absolute ethanol. Quenching corrections were made by the internal standard technique. All samples were counted for a sufficient period of time to achieve a 95% level of accuracy.

Preparation of Tritiated Androsterone Glucuronoside

Two mg of androsterone glucuronoside was sent to the New England Nuclear Corp. for random tritiation by exposure to tritium gas (Wilzbach, 1957). Upon its return, the material was dissolved in 10 ml of 0.1 M $NaHCO_3$ solution and allowed to stand at room temperature for 18 hours in order to exchange labile tritium. One mg of carrier androsterone glucuronoside was added and the solution was extracted twice with 15 ml of diethyl ether. The organic extract was discarded and the conjugate was recovered by acidification of the aqueous solution to *p*H 2 and by extracting four times with 10 ml of ethyl acetate. The ethyl acetate extracts were combined, washed twice with 5 ml of water, dried over Na_2SO_4, and finally evaporated to dryness in a flash evaporator at 40°.

The radioactive androsterone glucuronoside was purified by paper chromatography in the system ethyl acetate–hexane–acetic acid–water, 600:400:300:700 (v/v/v/v) as previously described (Schneider and Lewbart, 1959). The Zimmermann-positive region was eluted from the paper by shaking small segments in methanol for 3 hours. The paper was removed by filtration and the methanol was evaporated in vacuo.

A 1.0 × 25–cm column, containing 10 g of Celite, was prepared in the usual manner with solvent system K-G. Three mg of nonradioactive androsterone glucuronoside was chromatographed prior to running the radioactive sample. A 10% aliquot of each fraction was taken to dryness for assay of Zimmermann-positive material. A single peak was found whose maximum was present in fraction number 44, or 5.5 hold-back volumes.

Two mg carrier androsterone glucuronoside was then added to the radioactive material eluted from paper and the mixture dissolved in 1.0 ml of mobile phase and applied to the same column. The chromatogram is shown at the top of Figure 1 where it may be seen that only one Zimmermann-positive peak was eluted but that several radioactive impurities were separated. Those fractions which were Zimmermann-positive were combined and the solvents were removed by evaporation at 30°. It should be noted that a skewed peak was also obtained with the control androsterone glucuronoside sample. This nonideal behavior of androsterone glucuronoside was observed on other occasions in several different solvent systems.

The peak fractions from the previous column were rechromatographed on another 10-g column using system K-C. The results of this column are shown in the center of Figure 1 where it can be seen that an early peak of radioactivity was clearly separated from the Zimmermann-positive peak. The bulk of the androsterone glucuronoside peak was radiochemically homogeneous, as indicated by the good agreement between the radioactivity and Zimmermann curves. The average specific activity of these fractions was 18,100 cpm/μg. The material in those fractions indicated by the vertical lines was pooled and used as tracer androsterone glucuronoside in the subsequent experiments. The specific activity of an aliquot of the pooled material was 18,000 cpm/μg.

Proof of Radiochemical Homogeneity and Determination of Tritium Distribution of Tracer Androsterone Glucuronoside

Further evidence for the radiochemical homogeneity of the tracer androsterone glucuronoside was obtained in the following manner. An aliquot of androsterone glucuronoside with a radioactivity of 169,800 cpm was added to 9.00 mg of carrier androsterone glucuronoside and the mixture was crystallized from ethanol-water. The mother liquor was removed and taken to dryness, leaving a crystalline residue. Weighed samples of both crystals and mother liquor were then assayed for radioactivity. Values of 18,500 and 20,500 cpm/mg, respectively, were obtained.

In order to determine the distribution of radioactivity between the androsterone and glucuronic acid portions of the labeled molecules, tracer androsterone glucuronoside was hydrolyzed with perchloric acid and also by β-glucuronidase (Table I). Two aliquots of tracer androsterone glucuronoside were added to samples of nonradioactive carrier (1.783 mg in experiment A and 1.896 mg in experiment B). Each mixture was prepared as follows. Each mixture was dissolved in 10 ml of dry tetrahydrofuran containing 0.09 ml of 70% $HClO_4$. The solution was kept at 50° for 3 hours, after which it was cooled to room temperature and an excess of concentrated NH_4OH was added. The organic solvent was removed *in vacuo* and the residues were partitioned between 10 ml of water and 10 ml of benzene. The organic phase was separated, washed twice with 5 ml of water, and dried over Na_2SO_4. Aliquots of both the combined aqueous and benzene solutions were removed for radioassay. A larger aliquot of the benzene solution was taken to dryness and chromatographed on Celite in the system K-F. Only one symmetrical peak of radioactivity was eluted at 2.8–3.2 hold-back volumes. The fractions comprising the main peak of radioactivity were combined, the solvents were removed, and aliquots were taken for counting of radioactivity and Zimmermann analysis. The total amount of radioactivity contained in the androsterone moiety of the conjugate was calculated by multiplying the specific activity of the purified free androsterone by the weight of androsterone present in the unhydrolyzed mixture of androsterone glucuronoside. The percentage of tritium in androsterone was then calculated from this value and the initial value of total radioactivity in the conjugate. The data from experiments A and B are shown in Table I.

In a third experiment (C), the relative amount of organic solvent–soluble radioactivity obtainable from the tracer androsterone glucuronoside was also determined by incubation with β-glucuronidase. An aliquot with a radioactivity of 141,000 cpm was added

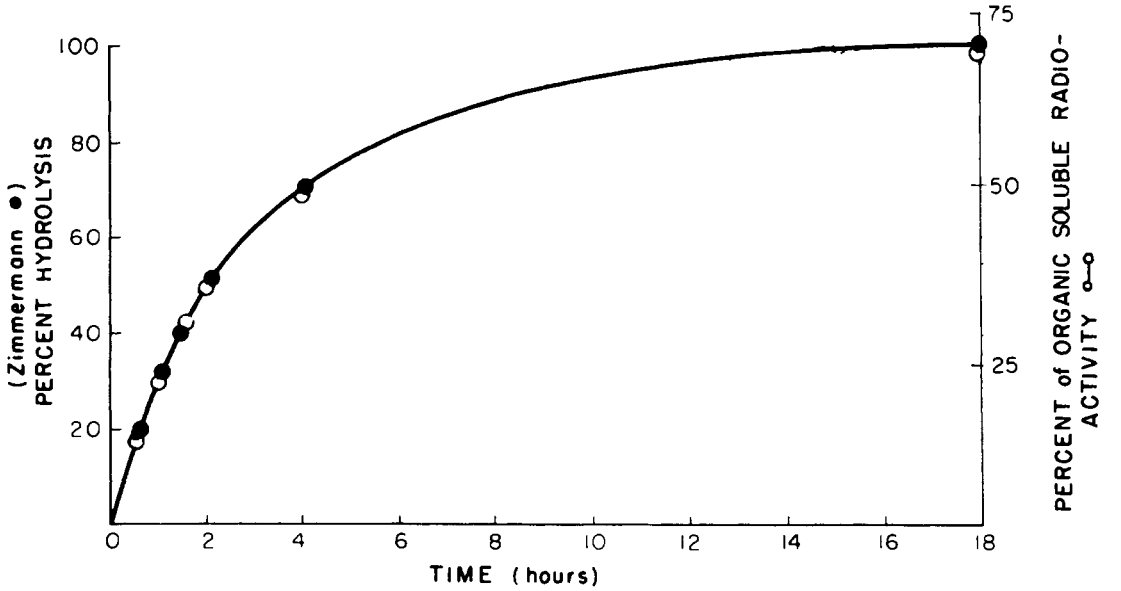

FIG. 2.—Hydrolysis of H^3-androsterone glucuronoside by β-glucuronidase. Per cent of initial radioactivity and Zimmermann values extractable by methylene chloride are plotted against time. See text for details.

to 2.00 mg of nonradioactive androsterone glucuronoside and the mixture dissolved in 8.0 ml of 0.1 M sodium actetate *p*H 5.0 buffer. Two ml of Ketodase (5000 units/ml) was added and the mixture was incubated at 37°. One-ml aliquots were removed at various intervals and boiled for 1 minute, and 4.0 ml of methylene chloride was added. The tubes were shaken well and centrifuged for 2 minutes, and duplicate aliquots of the methylene chloride solutions were removed for both Zimmermann and radioactive assay. A zero-time control without enzyme and an aliquot boiled for 30 minutes in 10% HCl were treated similarly. Figure 2 shows the results of both measurements plotted as percentage of initial values against time. It can be seen that 100% of the Zimmermann-positive material but only 71.4% of the radioactivity were recovered after 18 hours as methylene chloride-soluble material. Of interest is the fact that although identical Zimmermann assays were obtained for the 18-hour enzyme- and HCl-treated samples, only 50% of the initial radioactivity was extracted by solvent from the latter. Thus, approximately one third of the tritium in the labeled androsterone was removed by the acid treatment in aqueous media. This seems to indicate that treatment of tritiated steroids prepared by the Wilzbach procedure with acid at boiling temperatures may result in loss of radioactivity and may invalidate results obtained with these tracers.

From the experiments summarized in Table I the mean value for the percentage of tritium contained in the androsterone portion of tracer androsterone glucuronoside was calculated to be 71.6%. These experiments also provided further evidence for the radiochemical homogeneity and identity of the tracer sample with nonradioactive androsterone glucuronoside since the only radioactive steroid detected after hydrolysis was androsterone. Furthermore, the substrate behavior of the labeled conjugate towards β-glucuronidase was identical with that of nonlabeled material.

In vivo Experiments with Tritium-labeled Androsterone Glucuronoside

Although three experiments were carried out, only one (experiment 1 in Table II) will be described in detail since the results from each were similar. Since the tracer androsterone glucuronoside had been stored at 4° for approximately 6 months between the time of its preparation and use in this experiment, the material was rechromatographed in system K-G in order to insure radiochemical homogeneity. The results of this chromatogram are shown at the bottom of Figure 1. The peak fractions were combined and solvents were removed as usual. An aliquot with a radioactivity of 805,000 cpm was dissolved in 10 ml of 10% aqueous ethanol and administered intravenously to a normal male subject. Urine was collected for three days and kept frozen until processed. One-half of the urine was used for the isolation of androsterone glucuronoside and one-tenth was used for a reverse isotope dilution experiment.

Isolation of Urinary Conjugates.—Fifty per cent (w/v) of solid $(NH_4)_2SO_4$ was added to the urine, which was then extracted three times with one-half volume of an ether-ethanol (3:1, v/v) mixture. The organic extract was concentrated in a flash evaporator until an aqueous suspension whose volume was approximately 150 ml was left. In order to remove $(NH_4)_2SO_4$, the suspension was then extracted three times with 100 ml of normal butanol which had been saturated with water. The butanol extract was then evaporated to dryness leaving an oily residue weighing about 3.0 g. Since no attempt was made to quantitate this part of the experiment, the following step was added in order to reduce the weight of the extract and thereby facilitate subsequent chromatography. The residue was redissolved in 200 ml of normal butanol and the solution was extracted twice with 50 ml of 0.1 N NaOH and twice with 50 ml of water. The butanol was again evaporated to dryness leaving a residue which weighed 280 mg and had a radioactivity of 260,000 cpm.

The residue was dissolved in stationary phase and chromatographed on a 2 × 42-cm column, prepared from 86 g of Celite, using solvent system K-G. One hold-back volume amounted to 150 ml and fractions of 13.5 ml were collected. Figure 3 shows the results of analyses of aliquots of each fraction for Zimmermann-positive materials, sulfate esters, and radioactivity. It can be seen that two Zimmermann-positive peaks were separated, the first of which also contained sul-

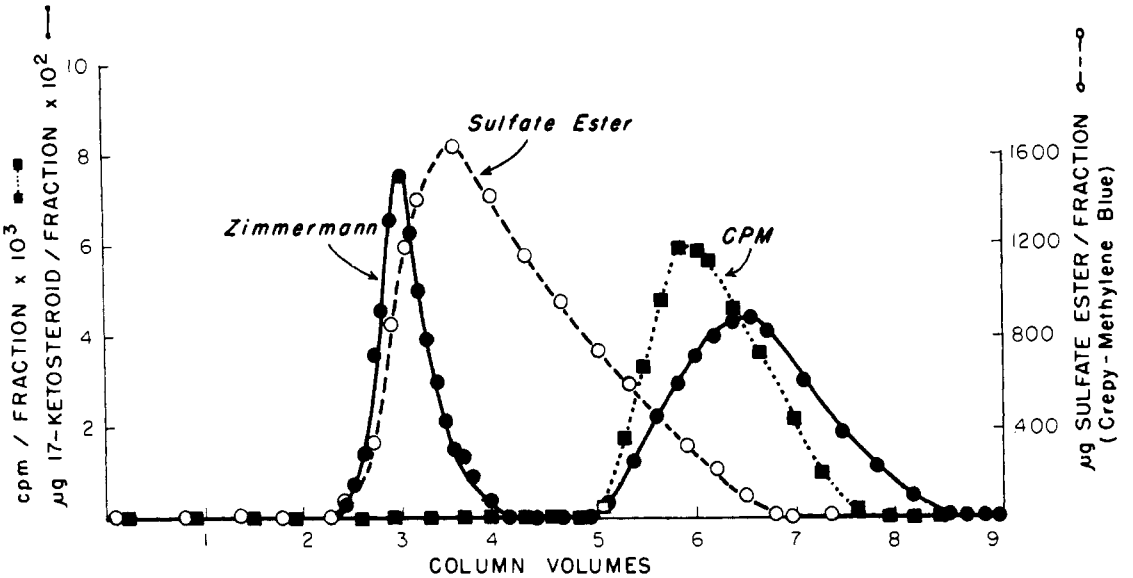

FIG. 3.—Column partition chromatographic separation of 17-ketosteroid sulfates and glucuronosides using system K-C. See text for details.

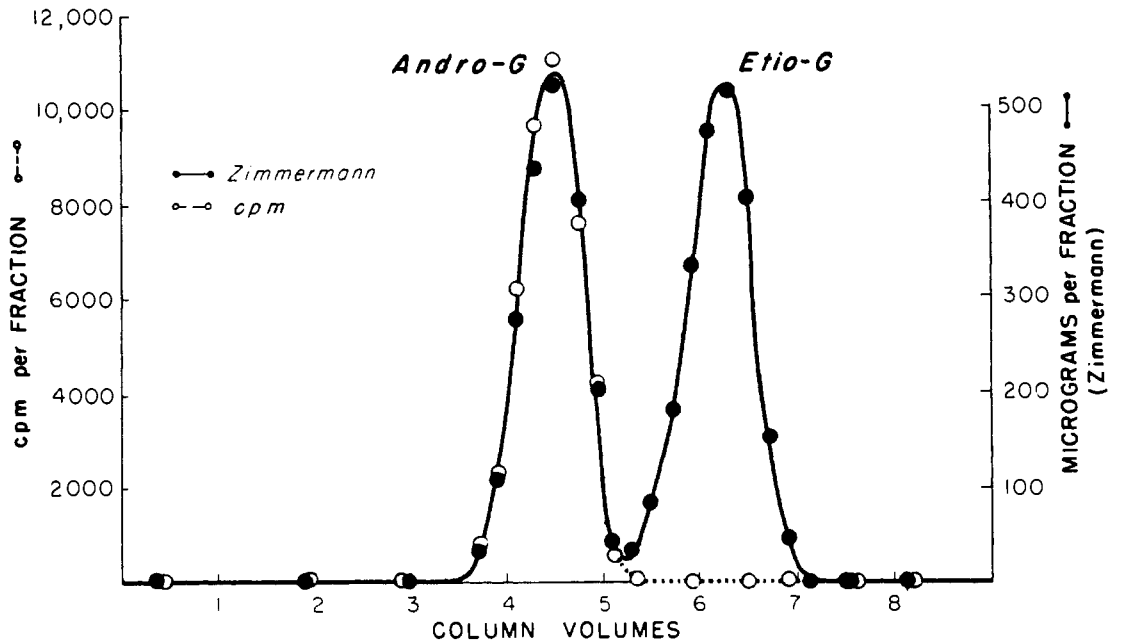

FIG. 4.—Column partition chromatographic separation of urinary 17-ketosteroid glucuronosides using system K-G. Andro-G = androsterone glucuronoside, Etio-G = etiocholanolone glucuronoside. See text for details.

fate esters but no radioactivity. The second, broad peak contained radioactive material in a position where androsterone glucuronoside would be expected (see Figure 1). Those fractions containing radioactivity were pooled and a residue weighing 55 mg was obtained upon removal of solvents. The fractions comprising the first Zimmermann-positive peak were also combined, and after removal of the solvent a residue weighing 50 mg was left. No radioactivity was recovered in the methanol wash of the column.

The *glucuronoside* fraction, with a radioactivity of 198,000 cpm, was rechromatographed on a 1.5 × 38–cm column of Celite (32 g) using solvent system K-G. Figure 4 presents the results of analysis of aliquots for Zimmermann-positive materials and radioactivity. It can be seen that two Zimmermann-positive peaks were again eluted, only the first of which contained radioactivity. The fractions comprising the two peaks were combined separately and the solvents were removed. The first peak material, weighing 5.4 mg, was rechromatographed in system K-G in order to remove colored impurities. However, several attempts to crystallize this urinary androsterone glucuronoside were unsuccessful. The material in the second peak was shown to be etiocholanolone glucuronoside, for when the material was hydrolyzed with β-glucuronidase the steroid liberated migrated in the Bush A system on paper in a manner identical with etiocholanolone. Crystallization of the unhydrolyzed etiocholanolone glucuronoside from dilute NaOH-ethanol yielded 2.1 mg of needles, mp 216–219°.

Radiochemical homogeneity of the urinary androsterone glucuronoside was indicated by the constant specific activity across the peak (see Figure 4). Since it was not possible to crystallize this material, its radiochemical purity was confirmed by crystallization

TABLE II
TRITIUM DISTRIBUTION IN ANDROSTERONE GLUCURONOSIDE ISOLATED FROM URINE

Experiment	Radioactivity Injected as Androsterone Glucuronoside (μc)	Tritium Distribution Present in Androsterone Moiety of Androsterone Glucuronoside (%)	
		Injected	Isolated from Urine
1	1.6	71.6	73.0
2	1.7	71.6	71.0
3	6.0	71.6	71.5

of an aliquot with a sample of unlabeled carrier. The specific activities of weighed samples of crystals and mother liquor, thus obtained, were 891 and 872 cpm μg, respectively. The specific activity of the undiluted androsterone glucuronoside isolated from urine as determined by Zimmermann assay and radioassay of appropriate aliquots, was found to be 23.3 cpm μg. Hydrolysis of this material by the $HClO_4$ acid procedure, as described above for tracer androsterone glucuronoside, showed that 73.0% of its radioactivity was still contained in the androsterone portion of the conjugate (see Table II).

The *sulfate* fraction from the first column (Figure 3) was hydrolyzed by the solvolysis technique and the free steroids were chromatographed on paper in the Bush A system. The area corresponding to androsterone was eluted with methanol and the eluate was dried. Zimmermann and radioactive assay of the crystalline residue indicated a specific activity of 0.55 cpm μg. The accuracy of this value is obviously questionable because of the low level of counts present. However, even if it were correct, this would correspond to approximately a 0.01% conversion of the administered androsterone glucuronoside to androsterone sulfate. The physiological significance of such a low conversion would be negligible.

Reverse Isotope Dilution Experiment.—Although the above data indicated that no cleavage of the urinary androsterone glucuronoside had occurred, it was necessary to ascertain whether the material actually isolated was representative of all the injected tracer. Therefore, the amount of radioactive androsterone glucuronoside present in the urine from experiment 1 was estimated by the reverse isotope dilution technique. For this purpose, 15.23 mg of nonradioactive androsterone glucuronoside was added to 10% of the 3-day urine collection and the mixture was treated with β-glucuronidase in the usual manner. Free steroids were extracted from the urine with two 500-ml portions of ethyl acetate. The combined organic extracts were washed twice with 200 ml of 0.1 N NaOH and twice with 200 ml of water, after which the ethyl acetate solution was dried over Na_2SO_4 and evaporated to dryness. An oily residue weighing 83 mg and having a radioactivity of 53,000 cpm resulted.

The residue was chromatographed on a 44-g column of Celite using solvent system K-F. Free androsterone and etiocholanolone were eluted at 4.2 and 5.2 hold-back volumes, respectively. Purity of the androsterone peak was indicated by the close agreement of the radioactivity and Zimmermann curves. The peak fractions were pooled, the solvents were removed, and the crystalline residue was recrystallized from ether-ligroin. The specific activity of a weighed sample of this product was 5.3 cpm μg. The total amount of radioactivity present as androsterone glucuronoside in the 3-day urine collection was then calculated by means of the following expression:

$$\text{total radioactivity in cpm} = \frac{a_1 \times a_2 \times w \times 10}{a_1 - a_2}$$

where a_1 = specific activity of the isolated androsterone glucuronoside, a_2 = specific activity of free androsterone adjusted to the weight of androsterone glucuronoside, and w = weight of androsterone glucuronoside carrier added to 0.1 of the volume of the urine. Substitution of the observed values 23.3, 4.6, and 15.23 for a_1, a_2, and w, respectively, yielded a value of 870,000 cpm. This calculated amount of urinary radioactive androsterone glucuronoside represents 107% of the injected dose of tracer.

The results from experiments 2 and 3 are also summarized in Table II. Tracer androsterone glucuronoside was administered to two other normal male subjects and the distribution of tritium in pure androsterone glucuronoside isolated from the subsequent urine collection was determined. In one experiment (No. 2) the tracer was infused over a 1-hour period and urine was collected at 2-hour intervals for 12 hours. Aliquots of each urine sample were dried in counting vials and assayed as described above. Although the accuracy of the counting method has not been established for urine samples, it was estimated that at least 90% of the injected radioactivity was excreted within 8 hours. The dose of injected radioactivity was increased approximately 4-fold in experiment 3. From the table it is evident that the percentage of tritium contained in the androsterone portion of the isolated conjugate from each experiment was the same as that of the injected tracer.

DISCUSSION

The fact that the distribution of tritium between the steroid and glucuronic acid portions of both the injected and urinary conjugate was the same in each of the three experiments described here constitutes strong evidence that the injected androsterone glucuronoside was not cleaved to the free steroid *in vivo*. The absence of significant amounts of radioactivity in urinary androsterone sulfate in these experiments is consistent with this conclusion. Nevertheless, this evidence alone is not unequivocal since it is conceivable that a fraction of the tracer androsterone glucuronoside was metabolically split and the liberated androsterone further metabolized to undetected compounds without prior mixing with the androsterone pool. However, the demonstration that all the injected radioactivity could be accounted for as urinary androsterone glucuronoside (experiment 1) appears to confirm the conclusion that neither the injected tracer nor the endogenously produced androsterone glucuronoside was further metabolized.

The same conclusion becomes evident if the production rate of androsterone glucuronoside is compared with its excretion rate. In experiment 1 it was possible to calculate the daily production rate (PR) of androsterone glucuronoside from the expression: PR = total radioactivity (in cpm) injected as tracer androsterone glucuronoside ÷ specific activity of urinary androsterone glucuronoside × days of urine collection. In this way the production rate was calculated to be 6.2 mg/day. An excretion rate of 5.3 mg/day was estimated from the amount of androsterone recovered from the urinary glucuronoside fraction and was not corrected for losses that may have been incurred during hydrolysis and isolation. Therefore most, if not all, of the endogenous as well as the administered androsterone glucuronoside was excreted into the urine unchanged during the experimental period. It thus appears that conjugation of androsterone with glucu-

ronic acid converts this steroid to a metabolic end product, which is destined for elimination from the body by the kidneys.

The observation that androsterone glucuronoside is not cleaved *in vivo* is somewhat surprising in view of the widespread occurrence of β-glucuronidase in mammalian tissue. Whether or not the formation of other naturally occurring steroid glucuronosides serves only as a means of eliminating steroid aglycones cannot be decided from the evidence presented here. For example, it is conceivable that other steroid glucuronosides may be more readily hydrolyzed *in vivo* by β-glucuronidase than is androsterone glucuronoside. In this connection the *in vitro* behavior of this conjugate towards enzyme hydrolysis (Figure 2) may be compared with that of dehydroisoandrosterone glucuronoside which we have found to be completely hydrolyzed by the end of 1 hour using identical conditions. Thus possible differences in susceptibility to enzymatic hydrolysis do not allow generalizations about the *in vivo* behavior of all glucuronosides.

Androsterone glucuronoside is rapidly cleared by the kidney, for although exact measurements were not made in this study, it was evident from experiment 2 that at least 90% of the injected radioactivity was excreted into the urine within 8 hours. The failure to detect *in vivo* cleavage of androsterone glucuronoside may in part be due to the fact that the bulk of the tracer was removed from the circulation by the kidneys before it could reach a tissue where further metabolism might have occurred. It has previously been demonstrated that androsterone glucuronoside as well as other 17-ketosteroid glucuronosides are more rapidly excreted than the corresponding steroid sulfates (MacDonald *et al.*, 1963). If the present results are related to the rapid removal of the injected conjugate, then it is likely that other steroid glucuronosides behave in a manner similar to that of androsterone glucuronoside.

The finding that androsterone glucuronoside is a metabolic end product is in marked contrast to the behavior of certain steroid sulfates which are freely interconvertible with their free steroids (Twombley and Levitz, 1960; Roberts *et al.*, 1961). Whether *in vivo* cleavage of sulfates is a general phenomenon or is restricted only to those steroidal compounds possessing some particular structural feature such as a phenolic or β,γ-unsaturated-sulfate group is not known. In order to answer this question, similar *in vivo* experiments are being carried out with labeled androsterone sulfate. The results will be published shortly.

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