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STUDIES ON PHENOLIC STEROIDS IN HUMAN SUBJECTS

XIV. STUDIES ON THE FATE OF INJECTED 6,7-H^3-OESTRIOL-3-C^{14}-GLUCOSIDURONATE

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SUMMARY

Labelled oestriol-3-glucosiduronate was administered to three human subjects in separate experiments: peripherally into a normal subject, peripherally into a subject with T-tube drainage, and peripherally with simultaneous instillation of 16-C^{14}-oestriol into the portal vein of a third subject.

Results show rapid excretion of the unchanged conjugate in the urine and no enterohepatic circulation. During this work, preliminary evidence is presented for the observation that in urine an equilibrium exists between oestriol-3-glucopyranosiduronic acid and its 3,6 lactone.

EVIDENCE for the existence of oestriol-3-glucosiduronate as a natural conjugate in humans was first presented by Troen *et al.*[1] and Wilson *et al.*[2], when this "polar conjugate" was detected in vital fluids of newborns and in pregnancy urine. Proof of structure of this compound was subsequently obtained by Goebelsmann *et al.*[3] upon isolating it from the urine of non-pregnant women to whom labelled oestriol and oestriol-16-glucosiduronate had been administered. The 3-glucosiduronate became readily available when Goebelsmann *et al.*[4] found that guinea pig liver homogenate catalyzed its formation from oestriol and uridine diphosphoglucuronic acid (UDPGA). Subsequently, Dahm *et al.*[5] reported that enzyme preparations of human intestine and placenta were capable of synthesizing the conjugate. The metabolic fate of oestriol-3-glucosiduronate infused simultaneously with the 16α-glucosiduronate into two pregnant women was investigated by Goebelsmann *et al.*[6]. These authors found that the former conjugate was excreted quantitatively and rapidly in the urine, and to a great extent unchanged, whereas the latter conjugate underwent hydrolysis (13%) and reconjugation *in vivo*. Støa and Levitz[7] found oestriol-3-glucosiduronate to be an intestinal metabolite of oestriol *in vivo* in humans. Inoue *et al.*[8, 9] found that 2–4 hr after terminating an infusion of 6,7-H^3-oestriol-C^{14}-glucosiduronate, there occurred urinary excretion of the 3-glucosiduronate with an elevated H^3/C^{14} ratio to the extent of 17% in 24 hr and continued excretion of the 16-glucosiduronate, but with a rapidly increasing H^3/C^{14} ratio. They also found that the distal segment of the human small intestine can synthesize this conjugate, if oestriol is available in that part of the alimentary tract.

In the present communication, results of three studies on the fate of oestriol-3-glucosiduronate in human subjects are presented. These results were obtained when (a) a mixture of 6,7-H^3-oestriol-3-glucosiduronate and oestriol-3-C^{14}-

glucosiduronate was infused intravenously into a normal woman, (b) a similar mixture was administered by single injection to a patient with a biliary fistula and (c) 6,7-H^3-oestriol-3-glucosiduronate was administered peripherally with the simultaneous injection of C^{14}-oestriol into the portal vein of a patient. These studies were carried out (a) to confirm earlier observations, by other workers, of rapid excretion in the urine of the oestriol-3-conjugate, (b) to ascertain whether the conjugating group remains unhydrolyzed when the conjugate is excreted, (c) to investigate any changes that may occur to the steroid moiety, (d) to demonstrate possible differences in enteric circulation and urinary excretion rates between oestriol and its 3-glucosiduronate and (e) to investigate the utility of previously developed methods [10–12] using countercurrent distribution (CCD) in separating metabolites, when volumes of urine larger (approximately 80 ml) than those previously used (10 ml) are fractionated.

The results of these experiments have confirmed the rapid and almost quantitative excretion of oestriol-3-glucosiduronate. It has been shown, also, that the conjugating group of the latter compound is not hydrolyzed, and that no detectable amount of the conjugate is excreted in the bile. It was not possible to detect any changes in the aglycone. In the third experiment (under (c), above) oestriol-3-glucosiduronate was excreted at a much faster rate than oestriol.

In the present work, the major portion of radioactivity present in the CCD train, upon separate distributions of aliquots of urine collected during experiments (a) and (b), was found in one symmetrical peak. However, on statistical fitting [10, 11] of the computed theoretical curves to the experimental data, these peaks invariably exhibited a wider base than could be accounted for by the presence of only one compound in the peak, and in all instances but one, two compounds per peak were estimated. This phenomenon, henceforth termed "peak broadening", was investigated in a preliminary manner. Results indicate that peak broadening occurs with urine extracts regardless of whether the compounds investigated were excreted in that medium or added to it. Peak broadening is due to the presence of more than one compound in the peak, but the change occuring to the conjugate is in the glucuronic acid moiety. This change is not of biochemical origin but is probably due to a different configuration of glucuronic acid and its existence in two or three forms at equilibrium. Detection of peak broadening is not possible without the use of recently developed mathematical treatment of CCD [10, 11].

MATERIALS AND METHODS

Singly-labelled oestriol-3-glucosiduronate was prepared according to the method of Goebelsmann *et al.* [4] by incubation of guinea-pig liver homogenate with either 6,7-H^3-oestriol (New England Nuclear, 50 μCi/μg) and unlabelled UDPGA (Sigma Chemical Co.) *or* unlabelled oestriol (Organon, Inc.) and uniformly labelled C^{14}-UDPGA (New England Nuclear, 0·213 μCi/μg). Purification was performed by distributing in a CCD apparatus (manufactured by Post Scientific Instrument Co.) the incubation mixture in the solvent system 0·2% ammonia: ethyl acetate: n-butanol; 4 : 1 : 3(v/v). Determination of the radioactivity in aliquots from the CCD tubes and estimation of the number of compounds in the CCD train, their partition coefficients (K) and amount of each as a percentage of the total, followed previously published [10, 11] procedures. Values of these parameters for the tritium-labelled material were as follows: 79% with a K of

0·32 and 21% with a K of 0·22 ($n = 99$). Estimations for the C^{14}-labelled material were as follows: 71% with a K of 0·01, 27% with a K of 0·34. Since oestriol-3-glucosiduronate has a K value of approximately 0·3 in the system used, the yield following incubation for the tritium-labelled compound was 79% and for the C^{14}-labelled material 27%.

Since it was important, for reasons that will become apparent below, that purity of the injected compounds be established to a high degree of certainty, the two preparations described above were separately re-distributed in the same system for 499 transfers. The H^3-labelled preparation was 99·5% pure and the C^{14}-labelled preparation was 99·7% pure.

Doubly-labelled oestriol-3-glucosiduronate was prepared by mixing the above two compounds to obtain the desired H^3/C^{14} ratio, as indicated in Results. Oestriol-16-C^{14} was a gift from Dr. Mortimer Levitz.

Prior to fractionation of urinary samples, the volume was reduced in a rotary evaporator (40°C) to approximately 10 ml. Techniques and instrumentation for the determination of radioactivity were identical to those reported previously [12]. Hydrolysis of conjugates was performed by incubation in 0·1M pH 5 acetate buffer for 48 hr at 37°C with 300 units/ml of β-glucuronidase (Ketodase).

RESULTS

Case A. E. P., a normal 23-yr old female, was infused intravenously for 5 hr with 500 ml saline solution containing a mixture of 5·4 μCi of 6,7-H^3 oestriol-3-glucosiduronate and 1·1 μCi oestriol-3-C^{14}-glucosiduronate ($H^3/C^{14} = 4·98$). Urine collections were made at 4, 8, and 12 hr after start of infusion. Results of the determination of excretion are given in Table 1. Recovery of radioactivity was quantitative, with no change in the H^3/C^{14} ratio. Recovery of more than 100% of the infused radioactivity is probably a reflection of small errors in determination of counting efficiencies, especially evident in double-label counting. The 1 ml aliquots of urine used in determining radioactivity, when dissolved in 15 ml ethoxyethanol solution of the scintillator [13], gave an external standard ratio of about 0·45. This is considered by us to be lower than the minimum (0·5) required for accurate determination of quenching, when factory prepared quench standards are used.

Half the volume of each urine collection was distributed to ascertain the nature of the excretory products. Results of these distributions are given in

Table 1. Urinary excretion of radioactivity following infusion* of a mixture of 6,7-H^3-oestriol-3-glucosiduronate and oestriol-3-C^{14}-glucosiduronate into subject E.P., a normal female

Hours†	Per cent of tritium injected	Per cent of carbon-14 injected	H^3/C^{14} ratio
4	50·4	48·8	5·14
8	52·7	51·5	5·10
12	3·5	3·3	5·41
Total	106·7	103·6	

*5·47 μCi of tritium and 1·10 μCi carbon-14 infused over a 5-hr period. H^3/C^{14} ratio of injected material = 4·98.

†From beginning of infusion.

Fig. 1, which is a combination graph and table, the format of which enables condensation of results from several distributions for clearer presentation. The results in this figure were obtained by determination of the disintegrations per minute (dpm) for the tritium and carbon-14 label in each tube of the CCD train after the distribution of a single collection. The dpm for each label were then computed *separately* to give the number of compounds in the train, as well as the percentages and partition coefficients of each. In these statistical computations, the 95% width of the confidence intervals is dependant upon variability of the data from the calculated ones. The standard deviation of counting (total counts accumulated) as well as the number of observations (such as width of peak, number of transfers, or whether each tube was counted) are factors affecting the confidence limits.

It was observed that in each of the urines examined, only one doubly labelled peak was present. Each peak has been computed to contain two compounds (arbitrarily designated *A* and *B*). The β value (K_{higher}/K_{lower}) for the two compounds is approximately 1·5 for the three urines. This β value is lower than the established resolving power ($\beta = 2$) for a conventional (vs. double) CCD. The ratios of *K* values estimated for the tritium and carbon-14 compounds for individual

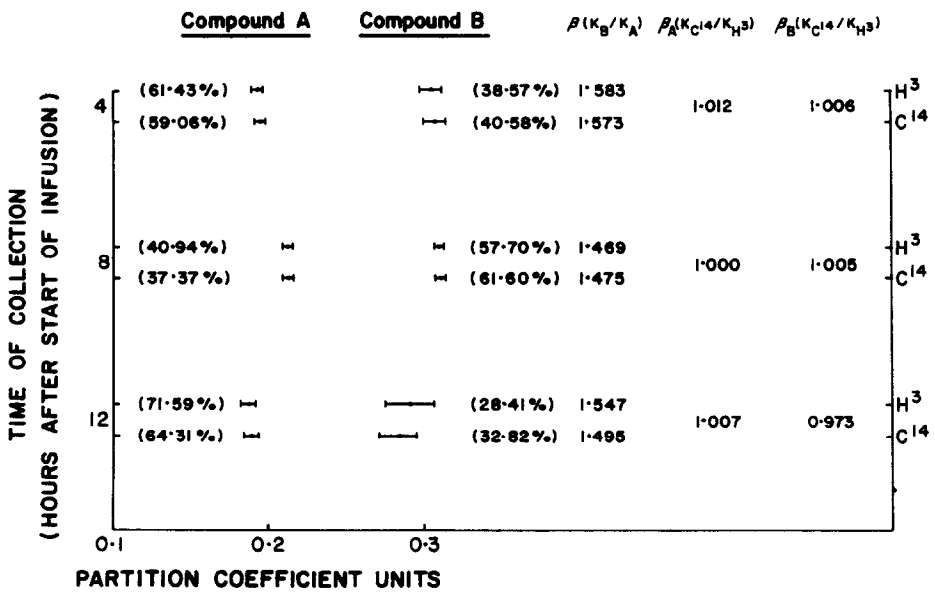

Fig. 1. Distributions of urine collections from subject E.P. after infusion of a mixture of 6,7-H^3-oestriol-3-glucosiduronate and oestriol-3-C^{14}-glucosiduronate. Half of the urine collected was distributed in each case ($n = 99$, system, 0·2% ammonia : n-butanol : ethyl acetate; 4 : 3 : 1 (v/v). Upon determination of the radioactivity in an individual distribution, the data (in dpm) for *each label* were computed separately to give the number of compounds in each curve as well as the percentage of *K* values. The horizontal bars in the figures are plots of the 95% confidence limits of the *K* values (represented by dots) for the computed compounds. Since there were only two compounds per distribution (minor ones of 2% or less were disregarded), the more polar compound was arbitrarily designated A and the less polar one B. *In all cases only one peak was observed in the CCD train.* The computed percentages of these compounds are given in parentheses. Two types of β values are given. One is derived by dividing the *K* values of compound *B* by that of *A* for a given label. The other is the ratio of *K* values for a given compound from the C^{14} and tritium curves. The confidence intervals are wider for the 12-hr distribution because the total amount of radioactivity was less than that of the 4- or 8-hr collections and, therefore, the data were not as precise.

distributions are given to show that computations for both curves consistently produced the same compounds. The very low β values thus obtained are within the range of those found[14, 15] for the isotope effect.

Overall, the K values estimated for compound A in these three distributions of Fig. 1 are similar, and the same observation holds true for compound B. The estimated percentages are inconsistent due to insufficient separation of compounds, as revealed by the β value of 1·5. It seemed necessary, therefore, to redistribute these excretory products for a larger number of transfers (n) required to achieve greater separation of these compounds. An approximate calculation for $n = 499$ showed that compounds A and B ($K = 0{\cdot}2$ and 0·3, respectively) should have peak tube numbers 80 and 120, respectively, within a bandwidth of 50 tubes. Accordingly, the peak from each urine was combined separately, the solvents were evaporated and the residues re-distributed for 499 transfers in the same system. Results of these distributions were anomalous. Instead of resolving the material into two peaks, there was no resolution and bandwidth had increased to approximately 80 tubes. Computations indicated three compounds per distribution, with correlation between the compounds in a distribution or between two corresponding compounds with different labels not as pronounced as in the case of the 99 transfer distributions above.

To gain insight into the causes of these anomalous results, the following hypotheses were investigated: (a) basic breakdown of estimation procedure, (b) bandwidth artifactually increased due to irregularities in distributions because of possible emulsion formation, and (c) some alterations in the conjugate molecules, most probably in the sugar moiety.

The experiments depicted in Table 2 were designed to investigate peak broadening. A 200 tube CCD instrument was used so that two separate 99 transfer distributions could be performed. In one distribution 5×10^5 dpm of 6,7-H^3-oestriol-3-glucosiduronate was treated in accordance with the various conditions described in the table and distributed, while in the other the same amount of conjugate was distributed without treatment and therefore was a "control" for the first. Computer estimates gave 96% or better of glucosiduronate in all "control" peaks.

Although the above experiments are by no means exhaustive, it was considered that the peak broadening phenomenon had been demonstrated to be due to a change in the biosynthetically prepared glucosiduronate upon being dissolved in urine. This conclusion has been recently corroborated by the independent work (though using the same methodology) of Slaunwhite and Roy[16, 17]. In this research, incubations of oestrone glucosiduronate with human placental homogenate did not cause peak broadening (metabolic changes did occur, but are irrevelant to the present study), while the same compound when injected into a human subject and collected in the urine underwent a change which was very similar to that reported above.

In order to locate the moiety of the conjugate in which the change has taken place, a portion of each of the solutes from the 499 transfer distributions referred to above of each urine collection from subject E.P. was hydrolyzed with β-glucuronidase at pH 5 (200 units/ml for 48 hr at 37°C., 0·1M acetate buffer). After incubation the volume was reduced and the solution made lower phase in tube 0 of the CCD train. Each of the three incubations was then distributed for 199 transfers in the system: methanol:water:ethyl acetate:heptane; 5:5:6:4

Table 2. Effect of various experimental conditions on peak broadening

Exp.	Experimental condition	K	%
1	Radioactive E_3-3-G1 was added to 80 ml fresh non-radioactive urine from a normal female. The urine was processed[1] and distributed in system a[2]	0·29	58·7
		0·21	41·3
	Control of above[3]	0·35	100·0
2	Solutions from the peak area of distribution 1 above were combined and redistributed in system a[2]	0·22	53·3
		0·32	45·9
		0·02	0·8
3	Radioactive E_3-16-G1 was added to 80 ml urine as in 1 above, processed[1] and distributed in system a[2]	1·74	68·4
		1·31	22·9
		2·08	8·8
	Control of above[3]	2·26	97·3
		1·29	2·7
4	Identical to 1 above, except for use of system b[4], pH 10–11	0·65	82·0
		0·84	13·0
		0·47	5·1
	Control for above, 80 ml of urine containing E_3-3-G1, treated as in 1 and distributed in system a[2]	0·22	56·7
		0·34	43·3
5	Radioactive E_3-3-G1 was added to 80 ml fresh non-radioactive urine from a normal male. The urine was dried in a rotary evaporator at 40°C. The residue was dissolved in the solvents from tube 0 of the CCD instrument, and the pH adjusted to 2 by addition of 1*N* HC1. The material was distributed in system C[5] for 99 transfers	2·08	88·4
		2·55	10·4
		0·97	1·2
		2·12	98·9
		0·86	0·6
	Control of above[3]	6·49	0·5
6	Radioactive E_3-3-G1 was added to 80 ml water, processed[1] and distributed in system a[2]	0·35	96·6
		0·05	1·2
		0·16	1·9
	Control for above[3]	0·36	97·0
		0·17	2·3
		0·07	0·7
7	Radioactive E_3-3-G1 was added to 80 ml fresh cooled urine, and the urine lyophilized and distributed in system a[2]	0·32	60·6
		0·24	39·4
	Control for above[3]	0·38	93·6
		0·31	5·0
		0·15	1·3

[1]Water was evaporated in a rotary evaporator under reduced pressure at a temperature of 40°C after addition of the radioactive material to the urine.

[2]System a: n-butanol : ethyl acetate: 0·2% ammonia; 3 : 1 : 4 (v/v).

[3]An identical amount of radioactivity distributed in the second bank of the CCD instrument. The radioactive conjugate was added to tube 0 of the instrument without any prior treatment to demonstrate that the untreated compound is pure.

[4]System b: n-butanol: 10% ammonia; 1 : 1 (v/v).

[5]System c: ethyl acetate: n-butanol: 0·01*N* HC1; 3 : 1 : 4 (v/v).

(v.v). Data from counting of the radioactivity were computed and the results are given in Fig. 2. It can be seen that hydrolysis was greater than 80%. The assumption that only one compound is present in the carbon-14 peak is not valid since that peak also contains unreacted glucosiduronate as well as free glucuronic acid. Because the system used is relatively non-polar, resolution of these extremely polar compounds is not expected. It can be confidently assumed from the results that the tritiated aglycone peak is comprised of one compound in each instance and is presumably oestriol.

Aliquots from the "oestriol" peak in each distribution were redistributed in the system: cyclohexane:ethyl acetate:ethanol:water; 5:5:4:6 (v.v) after mixing with 5 mg standard oestriol. Dpm and optical density thus obtained were submitted for analysis by the identification procedure[14, 15] and results are given in Table 3. For identification of high probability, three manifestations of statistical matching of K values from the radioactivity curve should be evident. First, the 95% confidence limits of the two partition coefficients should overlap;

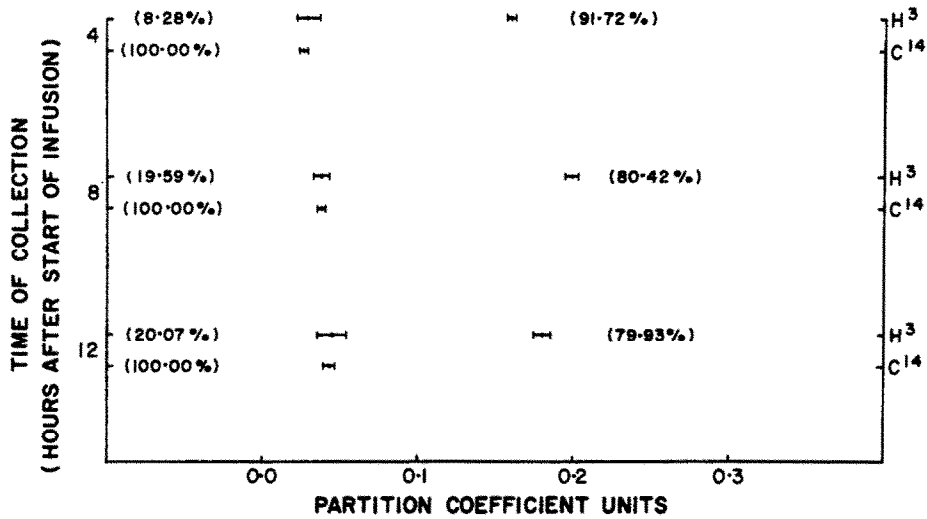

Fig. 2. Distributions of hydrolysate media from urine collections of subject E.P. See legend to Fig. 1 for explanation of format.

Table 3. Identification of the aglycone from urines of subject E.P.

Urine collection number	Partition coefficient (95% confidence intervals) Tritium	Carrier	$K_H3/K_{carrier}$	Peak tube separation	Percentages Carrier[1]	Tritium[2]
1	0·636 (0·625–0·646)	0·650 (0·645–0·655)	1·0223	0·52	100·00	76·93
2	0·685 (0·678–0·690)	0·677 (0·670–0·683)	1·0117	0·28	100·00	87·73
3	0·718 (0·714–0·722)	0·714 (0·709–0·719)	1·0055	0·13	98·48	65·27

[1]This column indicates the percentage of purity of the standard.

[2]This column indicates the percentage of the radioactivity in the tritium curve which can be attributed to the presence of a compound having the same K as the standard.

second, the ratio of K values (β value) should be small ($\leq 1{\cdot}02$) and third, the percentage of radioactivity in the tritium curve which can be attributed to the presence of a compound having the same K as the standard should be reasonably high ($\geqq 75\%$). It can be seen that identification of estriol was achieved in all three urine extracts.

Case B. A. L., an 83-yr old woman with a T-tube was injected three days after cholecystectomy with 5·4 μCi of 6,7-H^3-oestriol-3-glucosiduronate and 1·11 μCi oestriol-3-C^{14}-glucosiduronate (H^3/C^{14} ratio = 4·87). Urine and bile were collected each hour for 4 hr and urine was collected alone thereafter at 8 and 12 hr after administration. The urinary excretion of radioactivity is given in Table 4. As in case A above, excretion was rapid and quantitative within the first 8 hr and there was no change in the H^3/C^{14} ratio. No radioactivity could be detected in the bile of this subject. Figure 3 gives the data from distributions of aliquots from the first four urine collections ($n = 199$, system: 0·2% ammonia : n-butanol : ethyl acetate; 4 : 3 : 1 v/v). Peak broadening was also evident here, but note was especially made of the presence of small amounts of radioactivity in the polar region of the CCD train ($K \leq 0{\cdot}1$). The finding of some carbon-14 label in this area prompted the examination of the H^3/C^{14} ratio in each tube of the four CCD separations. In Fig. 4 are given four plots of ratio vs. tube number. It can be seen that the ratios in the area of 0·3 partition coefficient units are equal to that of the injected material. At higher K values, the curve rises steeply because of absence of C^{14}. However, in the region of $\simeq 0{\cdot}1$ K units, the H^3/C^{14} ratio is remarkably similar in all four distributions and is about twice that of the injected material. Because of the amounts of radioactivity, more detailed examination of the compounds was not undertaken.

Aliquots from the major peaks of each distribution were hydrolyzed with β-glucuronidase. Carrier oestriol (10 mg) was added to the chloroform extracts of the incubation media and each mixture was distributed ($n = 99$) in one of two systems (Table 5): Urines 1 and 2 in heptane : ethyl acetate : methanol : water; 3 : 3 : 2 : 4 (v/v), and urines 3 and 4 in an identical system except for the substitution of cyclohexane in place of heptane. Table 5 shows that upon application of the identification procedure[14, 15] to the data, identification of oestriol was accomplished in all four cases.

Table 4. Urinary excretion of radioactivity following injection of a mixture* of 6,7-H^3-oestriol-3-glucosiduronate and oestriol-3-C^{14}-glucosiduronate into subject A. L., a T-tube patient

Hours	Per cent of tritium injected	Per cent of carbon-14 injected	H^3/C^{14} ratio
1	39·4	37·3	5·14
2	20·1	19·1	5·13
3	16·4	15·1	5·30
4	13·2	12·0	5·35
8	11·8	11·3	5·07
12	5·3	4·6	5·62
Total	106·2	99·4	

*5·40 μCi of tritium and 1·11 μCi carbon-14 injected. H^3/C^{14} ratio of injected material = 4·87.

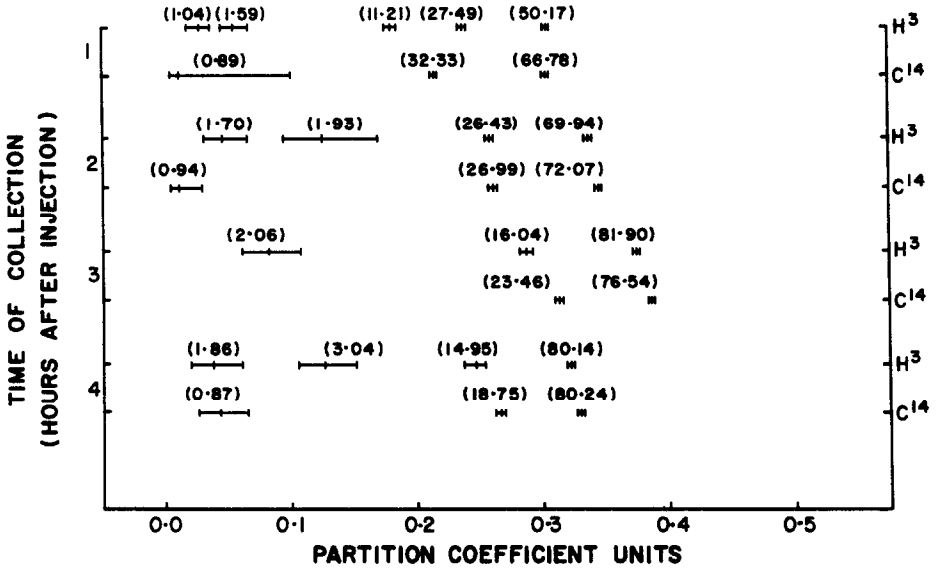

Fig. 3. Distributions of aliquots from the urine collections of subject A.L., a T-tube patient. See legend to Fig. 1 for explanation of format.

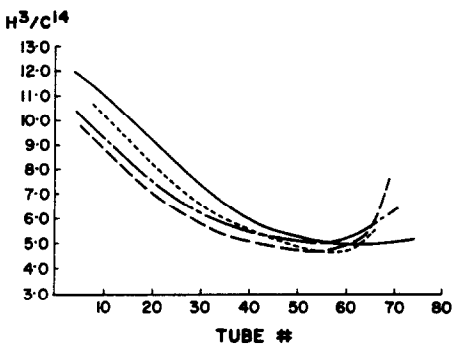

Fig. 4. Plots of the H^3/C^{14} ratios in the CCD tubes vs. tube numbers for each of the distributions in Fig. 3.

Table 5. Identification of the aglycone from urines of subject A. L.

Urine collection number	Partition coefficient (95% confidence intervals) Tritium	Carrier	$K_H3/K_{carrier}$	Peak tube separation	Percentages Carrier[1]	Tritium[2]
1	0·343 (0·333–0·354)	0·350 (0·345–0·355)	1·0195	0·38	100·00	73·00
2	0·340 (0·335–0·344)	0·344 (0·341–0·347)	1·0127	0·24	100·00	74·43
3	0·281 (0·274–0·281)	0·286 (0·285–0·287)	1·0174	0·30	90·83	88·32
4	0·297 (0·295–0·300)	0·298 (0·297–0·300)	1·0040	0·07	93·24	100·00

[1]See footnote 1, Table 3.
[2]See footnote 2, Table 3.

Case C. W. K., a 48-yr old woman with cancer of the cervix was injected intravenously during hysterectomy with 10 μCi of 6,7-H^3-oestriol-3-glucosiduronate into a peripheral vein and with 1·46 μCi of 16-C^{14}-oestriol into the portal vein. Excretion of radioactive material is given in Table 6. Two observations can be made. First, recovery falls far short of that observed in cases A and B. Similar observations were made by Inoue *et al.*[8, 9] and may be related to the stress of operation. Secondly, the difference in rate of excretion between estriol and its conjugate is very well documented by the change in H^3/C^{14} ratio. Excretion of radioactivity was very low after 8 hr. Distribution of aliquots from each urine in the system n-butanol:ethyl acetate: 0·2% ammonia; 3 : 1 : 4 (v/v) is given in Fig. 5. The tritium label is represented by one single major peak ($K = 0·25$). The remainder of the label, not shown in this figure, was represented in all three cases by a single peak of $K \geq 15$, and was not identified further.

Table 6. Urinary excretion of radioactivity following injection of 6,7-H^3-oestriol-3-glucosiduronate into a peripheral vein and C^{14}-oestriol into the portal vein of subject W.K.*

Hours	Per cent of tritium injected	Per cent of carbon-14 injected	H^3/C^{14} ratio
2	39·84	39·93	6·82
4	3·64	1·88	13·20
8	2·08	1·77	8·02
12	0·13	0·17	5·03
24	0·12	0·21	3·79
48	0·11	0·63	1·20
Total	45·92	44·59	

*10 μCi of tritium and 1·464 μCi of carbon-14 injected. Ratio = 6·83.

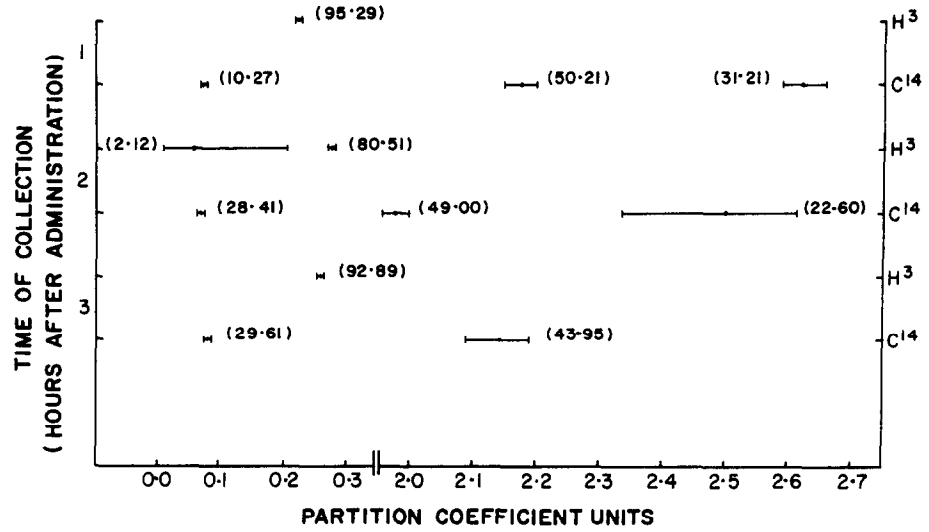

Fig. 5. Distributions of aliquots from the urine collections of subject W.K. See legend to Fig. 1 for explanation of format.

The carbon-14 label, originally injected as oestriol, now appears as two distinct peaks in each urine. The major one ($K \simeq 12$), is oestriol-16-glucosiduronate. This peak exhibited the broadening effect in all three urines. The minor peak is oestriol-3-sulpho-16-glucosiduronate. The relative amount of this compound increases with time, from approximately 10% in the first urine to 30% of the C^{14} label in the CCD train in the second and third urines. No effort was made in the present work to prove the structure of the compounds represented by peaks in Fig. 5. The metabolism of oestriol has been investigated extensively by several authors as well as in this laboratory. The percentage of sulphoglucosiduronate excreted conforms to that reported previously [8, 9].

DISCUSSION

One of the questions which the experiments described above were specifically designed to answer was, does oestriol-3-glucosiduronate remain intact upon instillation into the body, or is the conjugating group hydrolyzed and the aglycone reconjugated, as has been shown to occur for oestriol-16-glucosiduronate [6, 8]. From the literature, Goebelsmann *et al.* [6] infused a mixture of 15-H^3-oestriol-3-glucosiduronate and 16-C^{14}-oestriol-16-glucosiduronate into two *pregnant* women at midterm. Greater than 90% of the tritium label was excreted within the first 12 hr. Very little hydrolysis of the 3-glucosiduronate and reconjugation to the 16-glucosiduronate had taken place within that time, although a considerable change occurred later. However, this change is very small when considered in terms of the total excretion of radioactivity. More than 80% of the C^{14} label was excreted during that time. There was greater conversion of the 16- to the 3-conjugate (13%) than vice versa (2%). Inoue *et al.* [8] found that upon infusion of 6,7-H^3-oestriol-16-C^{14}-glucosiduronate, there was extensive hydrolysis of that compound, followed by reconjugation with glucuronic acid either at the 16-*or* the 3-position. Their evidence was based on an increased H^3/C^{14} ratio, but the ratios did not increase to infinity. This fact was an indication that some *but not all* of the C^{14}-glucuronic acid had been lost. To explain these results, transglucosiduronation, a phenomenon first discovered by Fishman and Green [18, 19] was invoked by Inoue *et al.* [8]. Fishman and Green [18, 19] demonstrated by *in vitro* studies the transfer of glucuronic acid from a donor conjugate (other than UDPGA) to a receptor molecule under unusual conditions of concentration. Therefore, while Fishman and Green [18, 19] postulated *inter*molecular transglucosiduronation, Inoue *et al.* [8] assumed *intra*molecular transglucosiduronation from position 16 to position 3. (Alternatively, the presence of a very small metabolic pool of glucuronic acid could be postulated).

The type of study described above can only be carried out with doubly-labelled conjugates, a technique first applied to steroid glucosiduronates in this laboratory [8]. It is essential that the purity of injected compounds and H^3/C^{14} ratios be carefully ascertained. Only recent advances in instrumentation as well as methodological innovations have made this possible. Specifically, the ease of obtaining reasonably accurate determinations of quenching in doubly-labelled samples for counting in scintillation spectrometers equipped with external standardization, and the computer-assisted statistical matching of calculated and observed curves from CCD, facilitates ascertaining the purity of radioactive conjugates found in tracer amounts. Mixing a singly-labelled conjugate with the same compound labelled with a different isotope makes possible the selection of

H^3/C^{14} ratios of the injected material at will, which is important for optimizing double-label counting.

Previous literature on the fate of injected oestriol glucosiduronates has indicated rapid and quantitative excretion in the urine. Sandberg and Slaunwhite [20] found nearly quantitative recovery ($\geqq$ 80%) of radioactivity in the urine following injection of C^{14}-oestriol-16α-glucosiduronate into a number of female subjects within 48 hr. No radioactivity could be detected in bile of T-tube patients.

In the present work, analysis of urines after administration of oestriol-3-glucosiduronate, either by single injection or by infusion, did not result in any discernible hydrolysis and reconjugation within the period of time in which excretion of radioactivity was completed. Evidence for this observation was found in the constant H^3/C^{14} ratios in successive urine collections (Tables 1 and 4). Excretion in cases A and B was complete within 8 hr, although, understandably, the *rate* of excretion (dpm/unit time) was slower in the infused subject, because infusion was continued for 5 hr. There was no biliary excretion of radioactivity in case B. This finding is similar to that of Sandberg and Slaunwhite [20] for the 16-glucosiduronate of oestriol, and may be related to the observation of Butler and Packhan[21] that glucuronic acid is "metabolically inert". The latter authors recovered labelled glucuronic acid that had been administered by intravenous injection into animals in the urine, without any conjugation.

The CCD statistical fitting procedure developed in this laboratory[9, 10] has been in routine use for a period of years by a number of investigators with very good results. Application in other than idealized situations has always been done with caution. High concentrations of solutes and the presence of interfering substances could cause gross departure from the binomial distribution with attendant invalidity of results.

One of the problems occasionally encountered during development of the procedure using test standards was a "peak widening" phenomenon. This was manifested in estimates of pure compounds which were divided into two imaginary compounds with very close K values and almost equal percentages as a result of peak widths slightly in excess of theoretical. The difficulty was solved by incorporating a "background parameter option"[13, 14] into the computer program. This background is *not* related to that used in the determination of radioactivity and may be due, when it occurs, to imperfect washing of the CCD glassware and/or the use of excessive amounts of tritium label in a previous distribution.

During the iterative computations, the background, which is subtracted from all observed data, starts at an initial high estimate and is continuously decreased concurrently with the changes in K and concentration parameters, until the preselected precision fit of observed and predicted curves is achieved. On inclusion of the background parameter, 100% estimates were correctly assigned peaks containing pure compounds, that had otherwise been computed to contain more than one substance. The reason is that the values of the observed data had been decreased by a very small but sufficient amount to "lower" the peak and cause a perfect fit with a theoretical curve for one compound.

The computations in Fig. 1 included background estimations. Therefore, these peaks are wider than could be accounted for by computing the proper background. The results in Table 2 of some preliminary experiments on this subject indicate that peak broadening is not an artifact due to imperfect distributions and other

experimental conditions (experiment 1 and control of experiment 4, Table 2). The effect is real, and there is a relation between peak broadening and dissolving the conjugate in urine. The effect does not take place in simple distributions (where solvents alone are used as shown by controls for experiments 1, 3, 5, 6, and 7, Table 2); if it did, it would be impossible to prepare pure conjugates using CCD. It is probable that peak broadening occurs because of changes in the glucuronic acid moiety of the conjugate. This is corroborated by the observation that this effect is less pronounced in mildly acidic (experiment 5) and basic (experiment 4) media. There are several reactions that can be postulated to take place, the most plausible of which is the free acid-lactone equilibration.

Imai and Hirasaka[22] have examined in detail the equilibrium in solution between glucuronic acid and its lactone. Glucurone is obtainable by heating an aqueous solution of glucuronic acid and crystallization from that medium. Dissolving *either* the acid *or* the lactone in water produces a mixture of both. This is evident by the change, with time, in optical rotary dispersion of the solution. The concentration of components at equilibrium is affected by temperature. For example, upon maintaining the temperature of a solution at 40°C for 150 hr, an equilibrium of acid and lactone is obtained in which the concentration of the latter is >30%. Mineral acids cause the equilibrium to be reached faster, without affecting the final concentrations. In the above example, equilibrium is attained after 12 hr only if acid or lactone is dissolved in 1*N* H_2SO_4.

While there is no direct evidence for the structure of the carbohydrate moiety of glucosiduronates *in solution*, examination of molecular models by the present authors engendered no fundamental reason why the facts found by Imai and Hirasaka[22] could not be applied to glucuronic acid conjugated to steroids.

In this laboratory[10, Table 3], it has been observed that biosynthesized oestriol-3-glucosiduronate has to be purified from a polar "contaminant". The β value of these two components is *ca.* 1·5. Inoue *et al.*[8] used $K \simeq 0{\cdot}2$ for the 3-glucosiduronate while we have used $K \simeq 0{\cdot}3$ in the same system (n-butanol: ethyl acetate: 0·2% ammonia; 3 : 1 : 4 (v/v). The ratio of these K values is also 1·5. The final step in preparation of glucosiduronate is evaporation of solvents from the combined solutions of the CCD tubes in the peak area. In all probability the organic solvents are predominantly removed first leaving a largely aqueous mixture.

From the arguments above, it can be postulated that the 3-glucosiduronate routinely prepared in this laboratory is in the γ-lactone form. Upon dissolution in urine, it is partially transformed to acid. Because of the long time required to reach equilibrium in water, it must be also postulated that urine catalyzes these transformations, and that in the CCD, we are dealing with a *reacting* system. This is corroborated by experiments 6 and 7 of Table 2 and by the percentages estimated for the various components of Figs. 1, 3 and 5.

The transformations of glucuronic acid postulated above probably do not affect either its biochemical or physiological functions when conjugated.

Figures 3 and 4 are given to show that there are small amounts of radioactivity in the lower K area of the CCD train ($K = 0{\cdot}10$), where double conjugates are located. The H^3/C^{14} ratios are similar in the three distributions and are higher than the ratio of injected material. To increase this ratio, C^{14} must be lost, i.e. some of the labelled 3-glucosiduronate must be hydrolyzed. To decrease the K

value concomitantly with increase in the ratio, one has to assume conjugation to more polar group(s).

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Note added in proof: Since this manuscript was approved for publication, Shimahara and Takahashi [23] have published a study of an infrared spectrophotometric study on the interconversion and hydrolysis of D-gluco-ν-and-δ-lactone in which they prove that intermediacy of gluconic acid is not necessary.