BBA 3846

GLUCURONIDE FORMATION IN THE TRANSPORT OF ESTRADIOL BY RAT INTESTINE *IN VITRO*

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(Received July 4th, 1962)

SUMMARY

When [^{14}C]-17-β-estradiol is incubated with everted sacs of rat intestine, net transport of radioactivity to the serosal medium is accomplished by oxidation of the estradiol to estrone followed by conjugation of the estrone as a glucuronide. The conjugated estrone is accumulated in the serosal medium. Relative impermeability of the mucosa to the conjugate offers a reasonable basis for the accumulation of radioactivity in the serosal fluid.

INTRODUCTION

It has recently been observed that glucuronide conjugates are formed during the transport of certain amino acid and thyroxine analogues by rat intestine *in vitro*[1,2]. When ^{131}I-labeled analogues of thyroxine are incubated *in vitro* with everted sacs of rat intestine, all the radioactivity in the serosal fluid is in the form of a glucuronide conjugate of the analogue. The glucuronides of these thyroxine analogues are not themselves transported by the everted gut sacs, and it has been suggested that relative impermeability of the mucosal surface of the gut to these conjugates offers a reasonable explanation for their accumulation in the serosal fluid.

To determine whether or not this mechanism for transport might have more general application, the ability of everted gut sacs to transport a number of steroids has been examined. [^{14}C]-17-β-Estradiol was chosen as a model estrogen. With this compound, net transport of radioactivity to the serosal medium is accomplished by a two-step process: the estradiol is first oxidized to estrone, and the estrone is conjugated as a glucuronide. The accumulation of this conjugate in the serosal fluid results in levels of radioactivity as much as 7 times greater than those in the mucosal medium.

EXPERIMENTAL

[16-^{14}C]-17-β-Estradiol (specific activity 5.5 μC/mg and 25 μC/mg) and [16-^{3}H]estrone (specific activity 250 μC/mg) were obtained from New England Nuclear Corporation. No radioactive impurities were detected when the chromatographed compounds were scanned in a standard gas flow strip counter.

Wistar strain male rats weighing 150–250 g were fasted 20–30 h, decapitated, and the intestine removed and rinsed in modified Ringer's solution. The intestine was everted and tied into weighed sacs approx. 3 cm in length according to the method of WILSON AND WISEMAN[3]. Except as noted in individual experiments, each sac was filled with 0.5 ml of medium containing 0.134 M NaCl, 0.011 M KCl, 0.008 M phosphate buffer (pH 7.4), 0.001 M $CaCl_2$, and 0.02 M glucose, and placed in a 20-ml beaker containing the same medium plus [16-^{14}C]-17-β-estradiol ($2 \cdot 10^{-6}$ M or $9 \cdot 10^{-6}$ M). The sacs were incubated for 120 min at 37° in a Dubnoff shaker oscillating 120 times/min with oxygen flowing at 4–5 cubic feet/h. At the end of the incubation, 0.2-ml aliquots of the inner and the outer fluid were placed in aluminum planchets and counted with a Geiger–Müller tube. Sample counts generally exceeded 10 times the background. Transport was calculated either as the difference between mucosal and serosal media in counts/min/100 mg wet weight of tissue/120 min or as the ratio of serosal to mucosal counts/min/unit volume. Gut sacs were taken from various segments of the small intestine; when inhibitors were used every second or third successive sac served as control. Chromatography of the mucosal and serosal media was performed on Whatman No. 1 filter paper in standard glass chromatographic tanks using Bush B_1, B_3, B_5 or Eberlein–Bongiovanni (E_2B) solvent systems[4,5]. Scanning of the labeled compounds was performed with a gas flow strip counter. Color reactions of the unlabeled estrogens were carried out with standard Zimmermann and phosphomolybdic acid reagents[4].

In experiments where the conjugate was made in quantity, successive sacs 5 cm in length were taken from the lower small intestine of 6 animals. The sacs were prepared in the standard manner except for being filled with 1 ml of unlabeled medium. Following 3 h of incubation, the serosal media were combined and evaporated under vacuum to a volume suitable for application to chromatography paper. After drying, the strips were equilibrated over night in standard Bush systems, then allowed to drip for 12–24 h, the water-soluble conjugate remaining at the origin. Following this, the paper was thoroughly macerated and the conjugate extracted with water. Hydrolysis of the conjugate was performed with "Ketodase" (Warner–Lambert) 1000 units/ml during a 3-h incubation in 0.5 M acetate buffer (pH 5) at 37°. Inhibition of β-glucuronidase activity was accomplished in control vessels with 0.001 M 1,4-saccharolactone as described by LEVVY[6]. 2 % Mylase P (Nutritional Biochemicals Inc., Cleveland, Ohio) was incubated with the conjugate for 18 h in 0.1 M phosphate buffer (pH 6.2) at 50° as suggested by COHEN AND BATES[7]. 5 % Takadiastase (K and K Laboratories, Jamaica, Long Island, N.Y.) was incubated with the conjugate for 18 h in 0.1 M acetate buffer (pH 6.1) at 37° as suggested by ABBOTT[8]. Acid hydrolysis of the conjugate was accomplished by refluxing with 0.1 N HCl under toluene for 24 h (see ref. 7).

Partition chromatography of the unknown steroid recovered from the glucuronide conjugate was performed on a Celite column in an 0.8 N NaOH–benzene system as outlined by BAULD[9]. Radioactivity was measured in aliquots from each fraction obtained from the column. To detect the added unlabeled estrone 1.0-ml aliquots of each fraction were taken to dryness, 1.0 ml of 10 % solution of phosphomolybdate in methanol was added, and the mixture placed in an 80–90° water bath until the methanol evaporated. The dried material was redissolved in 5.0 ml of methanol and the absorbancy read at 610 mμ in a Coleman model 6A spectrophotometer. Over the range of

concentration used in this experiment (5–125 μg) estrone standards produced a straight line relationship.

RESULTS

Incubation of everted gut sacs in standard medium containing [^{14}C]-17-β-estradiol resulted in a concentration of radioactivity in the serosal medium up to 7 times greater than that in the mucosal medium at the end of the experiment. As shown in Table I, the mean ratio established with everted sacs of small intestine at 2 concentrations of estradiol was 3. The entire intestine from duodenum to colon possessed the capacity to accumulate radioactive material in the serosal medium (Fig. 1). Although the results in Fig. 1 suggest that the ileum is slightly more active than the rest of the intestine, results from experiments with 8 animals indicate no statistically significant difference between transport in the upper and in the lower small intestine.

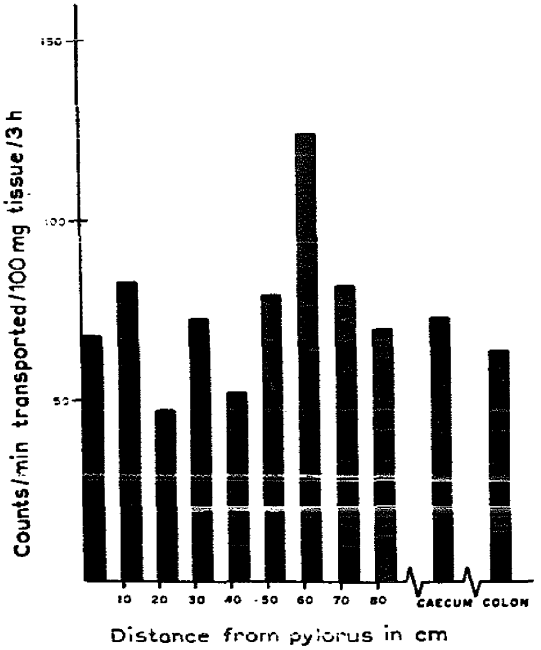

Fig. 1. Estrogen transport along the small intestine and colon.

The addition of various metabolic inhibitors to the medium and the incubation of sacs in an atmosphere of 100 % nitrogen markedly inhibited the transport of radioactive steroid into the serosal media, effectively preventing the development of a concentration gradient (Table II).

Paper chromatography of the post-incubation serosal medium indicated that the majority (from 60 to 95 % in different experiments) of the radioactivity was in the form of a water-soluble conjugate which remained at the origin in several solvent systems (Bush $\mathcal{B}_1$, Bush B_5 and Eberlein-Bongiovanni E_2B). The major portion of the remaining radioactivity chromatographed with the mobility of estrone. Less than 10 % of the original estradiol was still detectable. In the mucosal medium after a 3-h incubation, the majority of the radioactivity had a mobility similar to that of estrone.

TABLE I

ACCUMULATION OF RADIOACTIVITY IN SEROSAL MEDIA WITH [^{14}C]-17-β-ESTRADIOL

Concentration of estradiol	*Number of animals*	*Number of sacs*	*Mean ratios (serosal/mucosal media) ± S.D.*
$2 \cdot 10^{-6}$ M	6	16	3.04 ± 1.1
$9 \cdot 10^{-6}$ M	2	20	2.93 ± 1.4

TABLE II

EFFECT OF VARIOUS METABOLIC INHIBITORS ON TRANSPORT WITH [^{14}C]-17-β-ESTRADIOL

Estradiol concentration, $2 \cdot 10^{-6}$ M or $9 \cdot 10^{-5}$ M.

Inhibitor	*Concentration (moles)*	*Number of sacs*	*Mean per cent control transport*
2,4-Dinitrophenol	0.0004	3	16
Mercuric chloride	0.01	4	5
Sodium azide	0.03	3	0
Sodium fluoride	0.02	4	31
Sodium cyanide	0.02	3	0
100% Nitrogen		9	0

Less than 20% of the radioactivity was at the origin (presumably the conjugate) and little or none was detectable as estradiol.

In sacs where very high gradients developed, essentially all the radioactivity in the serosal medium was in the form of a conjugate. Under circumstances where accumulation was prevented, as with the metabolic inhibitors, none of the steroid was conjugated. Development of a gradient of radioactivity was dependent upon conjugation.

Properties of the conjugate

The steroid conjugate was water-soluble and could be eluted easily from the chromatography paper. When isolated and incubated with everted sacs of small intestine the conjugate was not actively transferred into the serosal medium. In 2 animals, the mean ratio of radioactivity of serosal to mucosal media following a 3-h incubation was 0.53. Although the quantity of radioactivity used was so small that if 15% or less of the material had been hydrolyzed it would not have been detected, there was no chromatographic evidence that the conjugate was broken down in these experiments.

In order further to characterize the conjugate it was prepared in quantity from the combined serosal media of 72 sacs as described above. The isolated conjugate was stable when incubated for 24 h at 37° pH 1, 3, 6, 9 and 12 but was hydrolyzed by refluxing in 0.1 N HCl under toluene for 24 h. The conjugate was also cleaved by incubation for 3 h at 37° with β-glucuronidase. The cleavage of the conjugate with β-glucuronidase could be prevented by including in the medium 0.001 M 1,4-saccharolactone, a specific inhibitor of β-glucuronidase[6]. The conjugate was not altered by

incubation for 18 h with Takadiastase or Mylase P, both of which cleave sulfate conjugates[7,8]. On the basis of these findings it is assumed that the conjugate is a glucuronide.

Properties of the conjugated steroid

When the glucuronide isolated from the serosal media was hydrolyzed either by refluxing with acid or by incubating for 3 h with β-glucuronidase, all of the radioactivity was liberated in a single unconjugated steroid. The mobility of this unknown

TABLE III

CHROMATOGRAPHY OF UNKNOWN STEROID WITH KNOWN ESTROGENS

	Bush B_1 R_F	*Bush B_3* R_F	*Bush B_5* R_F
Unknown	71	50	90
Estrone	70	49	86
Estradiol	34	16	83
Estriol	0	0	9

steroid was compared with mobilities of unlabeled samples of estradiol, estrone, and estriol applied to parallel strips. The unknown labeled steroid had mobilities in Bush B_1, Bush B_3 and Bush B_5 solvent systems comparable to those of the unlabeled estrone (Table III). When the unknown was applied to the same strips to which standards had been added the mobilities of the unknown were identical to those of estrone in the 3 systems used (Bush B_1 (R_F 80), Bush B_5 (R_F 91), E_2B (R_F 64)). In these 3 systems the unknown was clearly distinguishable from both estradiol and estriol.

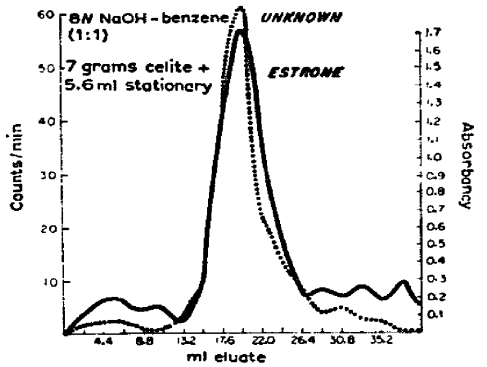

Fig. 2. Simultaneous partition chromatography of estrone and the unknown steroid in the aqueous sodium hydroxide–benzene system of BAULD[9].

Further support for the suggestion that the conjugated steroid is estrone was provided by partition chromatography in the aqueous sodium hydroxide–benzene system developed by BAULD[9] for the separation of estrone, estradiol and estriol. As noted in Fig. 2, when the unknown and standard unlabeled estrone were added simultaneously to the column the 2 substances were recovered in identical fractions of the eluate.

DISCUSSION

Although [^{14}C]-17-estradiol was used in most of these studies, [16-^{3}H]estrone is transported equally well by everted sacs of rat intestine. With estrone, the accumulation of radioactivity in the serosal medium is similarly dependent upon the formation of estrone glucuronide. The relative stability of the estrone glucuronide in alkali and in acid suggests that the conjugation is through the hydroxyl group at the third position rather than the ketone group at position seventeen[10].

The ability of slices of rat duodenum to form glucuronides of estrone and estradiol has been reported previously[11,12], but the experimental methods used would not have detected any interconversion of these estrogens. Although the methods of SCHURHOLZ AND STAIB[13] appear adequate for demonstrating the interconversion of estradiol to estrone prior to conjugation in the rat duodenum, none was demonstrated; estradiol was conjugated with glucuronic acid directly. In our preparations *in vitro* utilizing segments of the entire small intestine estradiol was oxidized to estrone prior to its conjugation. No direct conjugation of estradiol was detected.

Glucuronide formation offers a reasonable explanation for the active transfer of radioactive steroids across the intestinal wall in this preparation *in vitro*. Whereas the added steroids are soluble in lipid solvents and presumably in the tissue lipids, the conjugate is water-soluble. If it is assumed that conjugation occurs in the mucosal cells, the accumulation of the conjugate in the serosal fluid appears to result from a relative impermeability of the mucosal surface to the conjugate.

ACKNOWLEDGEMENTS

This investigation was supported by U.S. Public Health Service Grant A-1506, The Health Research Council of the City of New York, Contract U-1157, and by an Institutional Grant to Columbia University from The American Cancer Society.

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