# Evidence for the Absence of the Conjugates of Acne-Causing Hormones in Cow's Milk (36229)

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(Introduced by J. R. Spies)

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It is known (1–6) that the male hormones (androgens) and possibly progestigens stimulate the sebaceous glands, thereby leading to the development of acne. Since progesterone has been found (7–10) in bovine blood and tissues, especially those from pregnant animals, and androgens have been shown (11–13) to exist in bovine tissue as metabolites of progesterone, it is not surprising to find published speculations (14) that milk contributes to acne on the assumption that it contains large amounts of these hormones.

Data concerning the hormone content of milk are contradictory. Large quantities of 17- and 20-ketosteroids and hydroxy steroids have been reported to occur (15–17) in milk. Androsterone has also been identified (18) as one of many androgenic substances. Conversely, androgenic activity in milk was not found (19) using a bioassay method. Also, it was shown (20) that milk was of no importance in the excretion of progesterone and its metabolites after $^{14}C$-labeled progesterone was injected intravenously.

Because recent evidence obtained (21) in this laboratory indicated that the acne-producing hormones do not exist in milk fat and since it had been shown (22) in milk that hormones can be secreted via water-soluble conjugates; *e.g.*, glucuronides, sulfates, *etc.*, it was decided that the conjugated hormone content of cow's milk should be investigated.

*Materials*[1] *and Methods.* All chemicals were reagent grade and purchased commercially. Solvents were redistilled in glass.

*Isolation of steroid conjugates.* The procedure for the isolation of the conjugates was, with modification, that described by Bradlow (23). Approximately 300 g of the resin Amberlite XAD-2 (Rohm & Haas Co., Philadelphia, PA) was slurried in water and poured into a glass chromatographic column (50 × 4.8 cm i.d.) having a coarse fritted disc and a Teflon stopcock. The column was prepared for use by washing with 5–10 column volumes of demineralized water, 5–10 column volumes of methanol, and finally with 5 column volumes demineralized water. The column was maintained in water. Ten liters of raw skim milk (typical of market milk from mixed-herd, Agriculture Research Center, Beltsville, MD) was passed through the column at a flow rate of 50 ml/min. After all the milk had entered the column, the column was washed with ca. 5 liters of water or until the eluate was clear. The material adsorbed on the column was eluted using 4 liters of methanol. The methanol solution was evaporated to near dryness in a vacuum flash evaporator and the residue was dissolved in 50 ml of water. The water fraction was then extracted with 5 × 50 ml of chloroform, which was discarded.

*Hydrolysis of conjugates.* The hydrolysis of the conjugates was carried out similarly to the procedure used by Jones and Erb (24). The water-soluble material was adjusted to pH 4.5 with an acetate buffer, and 2 ml of chloroform were added. The mixture was then incubated with shaking at 37° for at least 24 hr with 20,000 units of β-glucuroni-

[1] The use of a trade name, distributor or manufacturer is for identification only and implies no endorsement of the product or its manufacturer.

dase (Calbiochem, Los Angeles, CA) containing sulfatase activity. After incubation, the free steroids were extracted with 3 × 50 ml of chloroform.

In order to hydrolyze more completely and also to hydrolyze other possible conjugates, the water solution was adjusted to pH 1 with sulfuric acid, treated with 10 g of sodium chloride and an equal volume of chloroform, and incubated at 37° for 24 hr. After the incubation period, the chloroform phase was removed and the water phase was extracted with 2 × 50 ml of chloroform. The chloroform extracts were combined, passed over anhydrous sodium sulfate (previously washed with redistilled chloroform) and then evaporated on a steam bath under a stream of nitrogen.

*Cleanup of extracts.* Eight percent (v/w) hydrated acidic alumina was prepared by the addition of distilled water to the dry alumina (Brockman Activity Grade 1, Baker Chem. Co., Phillipsburg, NJ). The mixture was shaken until all the lumps were broken and equilibrated overnight. Approximately 1 g of the alumina was placed in a Pasteur disposable pipette. The column was washed with 2 column volumes of 2% (v/v) methanol in chloroform and 2 column volumes of carbon tetrachloride. The combined residues were dissolved in ca. 200 μl of chloroform and the solution was carefully added to the column. The column then was washed with 3 column volumes of carbon tetrachloride, which removed some unwanted colored compounds, and then with 5 column volumes of 2% methanol in chloroform, which removed the hormones. The eluate was then evaporated to dryness on a steam bath under a stream of nitrogen.

*Gas–liquid chromatography (GLC).* The residue obtained was dissolved in 200 μl of chloroform, and 1 μl of the solution was analyzed by GLC. The instrument used was the Perkin-Elmer 900. The gas chromatographic column employed was a 3 ft × ⅛ in. i.d. stainless steel column packed with 3% (w/w) OV-1 (Silicone stationary phase, Applied Science Lab., State College, PA) on Gas Chrom Q (Applied Science Lab., State College, PA). The column was preconditioned at 280° for 48 hr with a helium flow rate of 50 ml/min. For the analyses, the column was programmed between 160 and 190° at 4°/min.

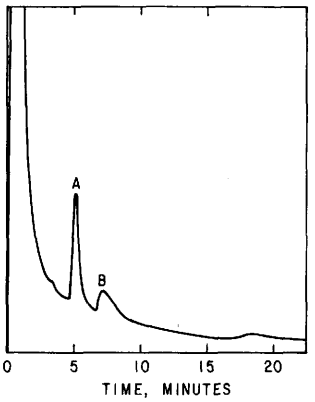


FIG. 1. GLC chromatogram of androsterone and estradiol recovered after adding their glucuronides to 10 liters of milk: (A) androsterone; (B) estradiol. Column: 3% OV-1, programmed 160-190° at 4°/min with helium flow rate of 50 ml/min.

*Recovery of glucuronides from milk.* Three commercially available glucuronides were used to evaluate the recovery capability of the method. Testosterone glucuronide and androsterone glucuronide (Supelco, Inc., Bellefonte, PA) were added to milk to give a concentration of 10 μg/liter. Estradiol glucuronide (Calbiochem., Los Angeles, CA) was added to give a concentration of 15 μg/liter. The glucuronides were found to be pure by thin-layer chromatography (TLC) on silica gel G (spots detected spraying with 50% conc sulfuric acid) and also by the method of Segura *et al.* (25).

*Results.* Figures 1 and 2 are typical chromatograms of the free hormones obtained after testosterone, estradiol, and androsterone glucuronide had been added to 10 liters of milk. The peaks obtained represent approximately 0.26 μg for testosterone, 0.22 μg for androsterone, and 0.16 μg for estradiol. Figure 3 shows the chromatogram obtained for 10 liters of milk only, under the same conditions. The recoveries, calculated by triangulation, were (on the av) 83% for testosterone, 70% for androsterone, and 54% for estradiol.

*Discussion.* As shown in Figs. 1, 2, and 3,

at the concentrations studied, the hormones were not detected in milk with the methodology used. The glucuronides, which are representative of the types of compounds expected, were easily extracted and their free sterol was identified. The size of the androsterone and testosterone peaks indicates that concentrations much less could be recovered and identified.

The lower recovery of estradiol is not easily explained. The hydrolysis was near 90% complete. One explanation is that the glucuronide of estradiol is either partly or totally the diglucuronide. However, TLC showed only one spot.

The question arises if the amounts of hormones needed to stimulate the sebaceous glands are below the concentrations studied. Methyl testosterone, one of the most potent stimulators of the human sebaceous glands, has been shown (3, 4) to produce physiological effects at the 5 mg/day level. But, controversy exists over the amount of progesterone needed. Some investigators have found (6) that 50 mg of progesterone, which is considered the physiological amount, taken each day for 12 weeks is needed to produce acne. Others have reported (2, 5) that even the physiological amount does not cause sebaceous gland enlargement. In addition, it has been reported (3) that dehydroisoandrosterone and $\Delta^4$-androstenedione are less potent stimulators than the testosterones. Therefore, it is apparent that milk does not contain these hormones as conjugates in amounts shown to be required for stimulation of the sebaceous glands.

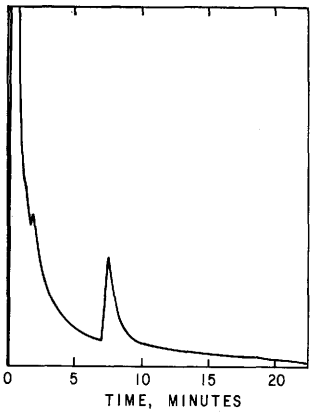


FIG. 2. GLC chromatogram of testosterone recovered after addition of its glucuronide to 10 liters of milk: conditions same as in Fig. 1.

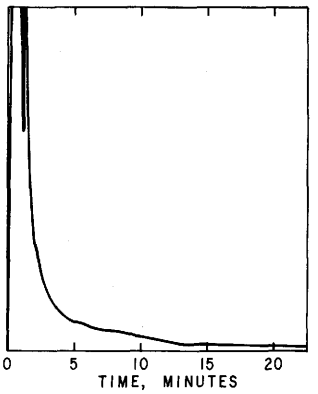


FIG. 3. GLC chromatogram of test for hormones in 10 liters of milk without addition of known glucuronides: conditions same as in Fig. 1.

*Summary.* Sex hormones known to produce acne were not detectable in bovine milk when analyzed by methods capable of detecting concentrations less than 10 $\mu$g of testosterone and androsterone and 15 $\mu$g of estradiol glucuronides/liter. These results show that ingestion of milk could not be the cause of acne on the basis of its conjugated hormone content.

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