The Oestrogenic Activity of Bovine Colostrum

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The oestrogenic activity of human colostrum (Lacassagne & Nyka, 1934) and the undoubted importance of colostrum for the neo-natal calf led us to test bovine colostrum for oestrogenic activity.

Oestrogens found in animal tissues and body fluids during the past 23 years, in particular those in pregnant-cow bile (Pearlman, Rakoff, Cantarow & Paschkis, 1947), have always been phenolic in chemical nature, although occurring partly as water-soluble, ether-insoluble derivatives (conjugates) in the case of some urines (Cohen, Marrian & Odell, 1936; Schachter & Marrian, 1938). We therefore assumed that oestrogens in colostrum would occur either as the free phenols or as derivatives yielding the phenols on acid hydrolysis; and in either case be extractable by boiling methanol.

EXPERIMENTAL

Material

Colostrum (9.5 kg. containing 2.9% fat, 20.5% solids-not-fat) was obtained on 19 February 1951 by the complete milking of the cow Pippin 12 of the Institute herd 3 hr. after calving, and was stored at -25° until required when it was rapidly thawed at room temperature.

Methods of extraction

Extraction of whole colostrum with hot methanolic NaOH was unsatisfactory owing to the difficulty of separating the small phenolic fraction from the large fatty-acid fraction. Direct ether extraction of whole colostrum at pH 7, pH 1, or when a large excess of concentrated HCl had been added was not possible because emulsions were formed.

(1) *Whole colostrum (free oestrogens). Bio-assay no. 1.* Whole colostrum (50 ml.) was refluxed for 10 min. with methanol (500 ml.), cooled to 30° and filtered. The residue was again refluxed with methanol (200 ml.) for 30 min. The combined filtrates were evaporated to dryness at reduced pressure, and saturated aqueous NaHCO_3 solution (100 ml.) and ether (100 ml.) added. After separation the NaHCO_3 phase was washed with more ether (6×100 ml.) and the combined ether solution, after drying over Na_2SO_4 , evaporated to dryness. The residue was dissolved in a mixture of CCl_4 (90 ml.) and ether (5 ml.), according to the method of Friedgood, Garst & Haagen-Smit (1948), and this solution extracted with portions of aqueous N-KOH (100, 50, 50 ml.). The combined alkaline solution was then brought to pH 1 with conc. HCl and extracted with ether (5×100 ml.). The ether solution was washed with saturated aqueous NaHCO_3 (2×10 ml.), dried over Na_2SO_4 and evaporated to dryness. The process was then repeated on a second portion (50 ml.) of colostrum and the two final products combined.

(2) *Whole colostrum (conjugated oestrogens). Bio-assay no. 2.* The combined NaHCO_3 solutions from process (1) above were brought to pH 1 with conc. HCl, mixed with conc. HCl (40 ml.) and boiled for 5 min. The cooled solution was then made slightly alkaline, brought again to pH 1 and extracted with ether (5×100 ml.). The ether solution was washed with several 10 ml. portions of water until these were neutral, dried over Na_2SO_4 and evaporated to dryness.

(3) *Clarified fat (free oestrogens). Bio-assays nos. 3 and 4.* Clarified fat (40 g.) obtained from whole colostrum (2 l.) by the method of Aschaffenburg *et al.* (1949) was dissolved in light petroleum, b.p. $60-80^{\circ}$ (200 ml.) and divided into two portions (50 and 150 ml.). The 50 ml. portion was extracted with N-KOH (4×50 ml.), the alkaline solution brought to pH 7 with conc. HCl and extracted with ether (6×50 ml.). This solution, dried over Na_2SO_4 , was evaporated to dryness (bio-assay no. 3). The 150 ml. portion was extracted similarly, the reagent and solvent volumes being trebled (bio-assay no. 4).

(4) *Clarified fat (conjugated oestrogens). Bio-assays nos. 5 and 6.* The final aqueous solution at pH 7 from the 50 ml. light petroleum of extraction (3) was brought to pH 1, freed of dissolved ether, mixed with conc. HCl (20 ml.), boiled for 5 min., cooled, made slightly alkaline, adjusted again to pH 1 and extracted with ether (7×100 ml.). This solution was washed with 10 ml. portions of water until they were neutral, dried and evaporated to dryness (bio-assay no. 5). The corresponding aqueous solution from the 150 ml. light petroleum of extraction (3) was similarly hydrolysed, quantities of reagents and solvents being trebled and the hydrolysis time increased to 15 min. (bio-assay no. 6).

(5) *Separated colostrum (quantitative estimation of oestrogens, free and conjugated). Bio-assays nos. 7 and 8.* Three 50 ml. samples of separated colostrum, prepared by a cream separator, were individually processed for free and conjugated oestrogens by the methods (1) and (2) (except that 20 ml. conc. HCl were used instead of 40 ml.). The three free-oestrogen concentrates were combined as were the three conjugated-oestrogen concentrates.

(6) *Separated colostrum (ketonic fraction of conjugated oestrogens). Bio-assays nos. 9, 10 and 11.* Separated colostrum (50 ml.), prepared by a cream separator, was processed to yield a free-oestrogen and a conjugated-oestrogen concentrate by the methods (1) and (2). The latter extract was then dissolved in ethanol (12 ml.) and acetic acid (1 ml.); Girard T reagent (0.5 g.) was then added and the solution refluxed for 30 min., cooled and added to ice-cold water containing Na_2CO_3 (0.88 g.) when the solution was yellow to bromothymol blue. The precipitate (P) was isolated by extraction with ether (5×50 ml.). The aqueous solution was then acidified with conc. HCl (12 ml.), left for 16 hr. at 20° and extracted with ether (5×50 ml.), this solution being washed with 10 ml. portions of water until they were neutral. This solution on drying and evaporation yielded ketonic fraction 1 (bio-assay no. 9).

Table 1. *Results of bio-assays for oestrogenic activity in bovine colostrum and normal milk*

Bio-assay no.	Source of material	Details of colostrum or milk specimen	Extraction method	Equivalent dose (ml. colostrum or milk/mouse)	Mean uterine weights (mg.)				Activity in dose ($\mu\text{g.}$ oestradiol- $17\beta/\text{l.}$)
					Test group	Oil-control group	0.02 $\mu\text{g.}$ Oestradiol- 17β group	0.04 $\mu\text{g.}$ Oestradiol- 17β group	
1	Pippin 12	Whole colostrum	{ Free oestrogens Conjugated oestrogens	10	6.3 $\pm$ 0.4	3.8 $\pm$ 0.6	9.4 $\pm$ 0.7	13.0 $\pm$ 1.5	1.0
2				10	21.2 $\pm$ 2.1	3.8 $\pm$ 0.6	9.4 $\pm$ 0.7	13.0 $\pm$ 1.5	> 8.0
3	Pippin 12	Clarified fat	{ Free oestrogens by KOH extraction	50	4.7 $\pm$ 0.3	3.5 $\pm$ 0.2	7.8 $\pm$ 0.5	15.2 $\pm$ 1.9	0.12
4				37	3.1 $\pm$ 0.3	3.8 $\pm$ 0.3	8.6 $\pm$ 0.6	14.0 $\pm$ 1.1	N.S.*
5	Pippin 12	Clarified fat	{ Conjugated oestrogens only	50	5.4 $\pm$ 0.3	3.5 $\pm$ 0.2	7.8 $\pm$ 0.5	15.2 $\pm$ 1.9	0.18
6				37	5.3 $\pm$ 0.2	3.8 $\pm$ 0.3	8.6 $\pm$ 0.6	14.0 $\pm$ 1.1	0.16
7	Pippin 12	Separated colostrum (cream separator)	{ Free oestrogens Conjugated oestrogens	15	4.5 $\pm$ 0.4	3.9 $\pm$ 0.5	6.5 $\pm$ 0.6	12.7 $\pm$ 1.4	0.13
8				1	3.9 $\pm$ 0.3	3.9 $\pm$ 0.5	6.5 $\pm$ 0.6	12.7 $\pm$ 1.4	N.S.*
9	Pippin 12	Separated colostrum (cream separator)	{ Conjugated oestrogens Non-ket. frac.	2	6.0 $\pm$ 0.4	3.9 $\pm$ 0.5	6.5 $\pm$ 0.6	12.7 $\pm$ 1.4	8.0
10				4	7.2 $\pm$ 0.5	3.9 $\pm$ 0.5	6.5 $\pm$ 0.6	12.7 $\pm$ 1.4	5.5
11	Pippin 12	Separated colostrum (cream separator)	{ 1st ket. frac. 2nd ket. frac.	8	12.8 $\pm$ 1.0	3.9 $\pm$ 0.5	6.5 $\pm$ 0.6	12.7 $\pm$ 1.4	5.0
12				5.0	5.6 $\pm$ 0.3	3.2 $\pm$ 0.5	8.8 $\pm$ 0.9	12.3 $\pm$ 0.7	1.8
13	Pippin 12	Separated colostrum (cream separator)	{ Conjugated oestrogens Non-ket. frac.	5.0	3.5 $\pm$ 0.4	3.2 $\pm$ 0.5	8.8 $\pm$ 0.9	12.3 $\pm$ 0.7	N.S.*
14				5.0	7.8 $\pm$ 0.7	3.2 $\pm$ 0.5	8.8 $\pm$ 0.9	12.3 $\pm$ 0.7	3.4
15	Normal milk	Free and conjugated oestrogens	—	20	4.4 $\pm$ 0.3	5.0 $\pm$ 0.6	10.4 $\pm$ 0.9	13.0 $\pm$ 1.5	N.S.*

* N.S. = activity not significantly different from zero ($P > 0.05$).

The Girard separation described was then repeated on the ether-soluble precipitate (*P*) above yielding ketonic fraction 2 (bio-assay no. 10) and a final non-ketonic fraction (bio-assay no. 11).

(7) *Normal milk (free and conjugated oestrogens)*. Bio-assay no. 12. Four 50 ml. portions of a sample of bulked milk from the Institute herd were individually processed for free and conjugated oestrogens by the methods (1) and (2), using 20 ml. conc. HCl for each hydrolysis. All eight concentrates were combined.

Method of bio-assay

Each final extract was dissolved in arachis oil and its oestrogenic activity estimated by the method of Evans, Varney & Koch (1941), except that the albino mice, from the closed colony of the Agricultural Research Council Field Station, Compton, Berks, were of 8–10 g. body weight, and six mice were used per group. The uteri, after dissection,

were fixed in Bouin's fluid for 72 hr., pressed dry on blotting paper and weighed.

RESULTS

Mean uterine weights of all test and control groups of mice are given in Table 1 with standard errors of the means.

The oestrogenic activity of each colostrum specimen has, for convenience, been expressed as $\mu\text{g. oestradiol-17}\beta/\text{l.}$, but only for no. 8, where it was possible to fulfil the conditions for strictly valid bio-assay (Emmens, 1948), could a quantitative estimate of the oestrogenic activity of separated colostrum be made, activity equivalent to $5.21 \mu\text{g. oestradiol-17}\beta/\text{l.}$ ($3.96\text{--}6.58 \mu\text{g.}$: 5% fiducial limits) being found. The analysis of variance of this estimation is given in Table 2. In Table 3 the results are summarized diagrammatically.

Table 2. *Factorial analysis of the dose-response line of conjugated oestrogens in separated colostrum*

(Cow: Pippin 12. Bio-assay no. 8, Table 1.)

Source of variation	Degrees of freedom	Mean square	Variance ratio	P
Difference between samples	1	0.735	—	N.S.*
Linear regression	1	208.86	38.95	$P < 0.01$
Departure from parallelism	1	0.482	—	N.S.*
Error	20	5.3682	—	—

* N.S. = activity not significantly different from zero ($P > 0.05$).

Table 3. *Summary of oestrogenic activity of colostrum of the cow Pippin 12*

(Values in $\mu\text{g. oestradiol-17}\beta/\text{l.}$)

Whole colostrum (free 1.0; conjugated > 8.0)			
Clarified fat (free N.S.*; conjugated 0.15)	Separated colostrum (free N.S.*; conjugated 5.2)		
	Ketonic fraction I (conjugated 1.8)	Ketonic fraction II (conjugated N.S.*)	Non-ketonic fraction (conjugated 3.4)

* N.S. = not significantly different from zero ($P > 0.05$).

Table 4. *Oestrogenic activity of body fluids*

	Body fluid	Oestrogenic activity ($\mu\text{g. oestradiol-17}\beta^*/\text{l.}$)	Reference
Bovine	Colostrum (free oestrogens)	1	Pope & Roy (this paper)
	Colostrum (conjugated oestrogens)	5	Pope & Roy (this paper)
	Pregnancy blood (free oestrogens)	4	Szego & Roberts (1946)
	Pregnancy bile (free oestrogens)	330	Pearlman <i>et al.</i> (1947)
	Non-pregnancy urine (free oestrogens)	1.1	Turner, Frank, Lomas & Nibler (1930)
	Pregnancy urine (free oestrogens)	50–600	Hisaw & Meyer (1929); Nibler & Turner (1929)
	Pregnancy urine (free and conjugated oestrogens; peak value)	1700	Barrie, Patterson & Underhill (1935)
Human	Colostrum (whole fluid injected)	10	Lacassagne & Nyka (1934)
	Pregnancy blood (free oestrogens)	6	Szego & Roberts (1946)
	Pregnancy urine (free and conjugated; peak value)	4000	Smith, Smith & Pincus (1938)

* The various results have, where necessary, been converted to these units for ease of comparison.

DISCUSSION

Table 4 shows that the oestrogenic activity of bovine colostrum is of the same order as that of human colostrum and of bovine and human pregnancy blood, but much lower than that of bile and urine in pregnancy.

The fact that most of the oestrogenic activity of bovine colostrum is found in the aqueous phase as water-soluble conjugates suggests that colostrum may serve as a vehicle for the excretion of small amounts of oestrogenic metabolites. These may, however, be significant in the health of the suckling calf. It is of interest that ketonic and non-ketonic conjugated oestrogens as found by us in bovine colostrum have also been reported in bovine blood and urine (Asdell, 1952).

The failure to detect oestrogenic activity in normal milk at a dose-level equivalent to 20 ml./mouse means that activity declines by a factor of at least 10 as the secretion changes from colostrum to normal milk. Lawson, Stroud & Williams (1945) also estimated the free oestrogens in normal cow's milk but obtained no response with doses equivalent

to 756 ml. in the ovariectomized rat, a finding which agrees with our results.

SUMMARY

1. Oestrogenic activity approximately equal in amount to that found in bovine and human pregnancy blood has been found in bovine colostrum.

2. The oestrogens occur mainly as conjugates in the aqueous phase of colostrum and are partly ketonic.

3. The conjugated oestrogens of separated colostrum from one cow were equivalent to 5.2 μ g. oestradiol-17 β /l.

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REFERENCES

- Aschaffenburg, R., Bartlett, S., Kon, S. K., Terry, P., Thompson, S. Y., Walker, D. M., Briggs, C., Cotchin, E. & Lovell, R. (1949). *Brit. J. Nutrit.* **3**, 187.
- Asdell, S. A. (1952). *Report of the 2nd International Congress of Physiology and Pathology of Animal Reproduction and of Artificial Insemination*, 2, 7.
- Barrie, M. M. O., Patterson, J. B. E. & Underhill, S. W. F. (1935). *Quart. J. Pharm.* **8**, 424.
- Cohen, S. L., Marrian, G. F. & Odell, A. D. (1936). *Biochem. J.* **30**, 2250.
- Emmens, C. W. (1948). *Principles of Biological Assay*. London: Chapman and Hall.
- Evans, J. S., Varney, R. F. & Koch, F. C. (1941). *Endocrinology*, **28**, 747.
- Friedgood, H. B., Garst, J. E. & Haagen-Smit, A. J. (1948). *J. biol. Chem.* **174**, 523.
- Hisaw, F. L. & Meyer, R. K. (1929). *Proc. Soc. exp. Biol., N.Y.*, **26**, 586.
- Lacassagne, A. & Nyka, W. (1934). *C.R. Soc. Biol., Paris*, **116**, 844.
- Lawson, W., Stroud, S. W. & Williams, P. C. (1945). *J. Endocrinol.* **4**, 83.
- Nibler, C. W. & Turner, C. W. (1929). *Proc. Soc. exp. Biol., N.Y.*, **26**, 882.
- Pearlman, W. H., Rakoff, A. E., Cantarow, A. & Paschkis, K. E. (1947). *J. biol. Chem.* **170**, 173.
- Schachter, B. & Marrian, G. F. (1938). *J. biol. Chem.* **126**, 663.
- Smith, G. V. S., Smith, O. W. & Pincus, G. (1938). *Amer. J. Physiol.* **121**, 98.
- Szego, C. M. & Roberts, S. (1946). *Proc. Soc. exp. Biol., N.Y.*, **61**, 161.
- Turner, C. W., Frank, A. H., Lomas, C. H. & Nibler, C. W. (1930). *Res. Bull. Mo. agric. Exp. Sta.* no. 150.