INTRAOVARIAN CONTROL OF FOLLICULOGENESIS: LIMITS TO SUPEROVULATION?

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INTRODUCTION

The ability to increase the ovulation rate in domestic animals by gonadotrophin treatment originated from experiments carried out more than sixty years ago (1). These seminal discoveries were however, rapidly followed by reports of excessive variability in the numbers of eggs shed in response to a standardized amount of injected hormone (2). Despite numerous attempts to overcome this problem in the intervening years little substantial progress has been made towards the objective of developing reliable methods for the hormonal stimulation of adequate and predictable numbers of embryos for transplantation. It is the objective of this paper to identify the hormonal and intraovarian factors which determine both the number and the quality of eggs shed after gonadotrophin stimulation. In addition, the probable limits to gonadotrophic stimulation of ovarian function will be discussed and methods for their circumvention outlined.

We shall suggest in the first section of the paper that the mechanisms that control follicle population dynamics dominate all others in determining the extent of superovulation in each animal. The interrelationship between these physiological regulators and superovulation can best be appreciated by considering the question of exactly how exogenous gonadotrophins increase the ovulation rate. The factors which determine the quality of oocytes shed after hormone injection will

be considered in the second section of this contribution. Particular emphasis will be placed on our recent studies, which show how different gonadotrophins alter to varying degrees the normal development of oocytes during maturation. New methods of avoiding the limitations imposed by superovulation will be outlined in the concluding section of the paper. The opportunity offered by this in vitro method for the production of large numbers of viable eggs from abattoir material will be of the utmost importance in the future improvement of livestock by genetic manipulation.

FOLLICLE POPULATIONS, GONADOTROPHINS AND MULTIPLE OVULATIONS

It is apparent that exogenous gonadotrophins increase the ovulation rate by activating responsive follicles. Of much less certainty, however, are the characteristics which endow certain classes of follicles with the capacity to respond to injected hormones. It is by examining the normal process of folliculogenesis and the modifications induced by superovulation that the practical limitations of this process can be understood.

a. Dynamics of follicular development during the bovine estrous cycle

The available evidence suggests that bovine follicles leave the non-growing pool and commence development at privileged periods during the cycle (3). Thereafter, growth is continuous until the follicle ruptures at ovulation or else undergoes atresia (4, 5). Despite the continuity of growth a number of intrafollicular factors including estradiol-17β ($E_2 17\beta$), inhibin and secreted proteins modulate the rate of growth in antral follicles (6,7,8). Estradiol output increases sharply as the bovine follicle reaches 8mm diameter (9); synthesis of this hormone is critical since it promotes follicular vascularization and thereby increases the uptake of gonadotrophins and other nutrients (10). In this way the most active follicle stimulates its own growth, whilst at the same time inhibiting growth and differentiation of other follicles by reducing, centrally or locally, the available levels of FSH and other gonadotrophins. Although circulating levels of FSH in the luteal phase are sufficient to stimulate follicle growth, they are unable to support the very largest follicles which consequently degenerate, thus enabling smaller follicles to grow.

The above concepts regarding folliculogenic control can be presented at a practical level by examining the distribution of follicles in the ovaries of 32 cows slaughtered at random during the estrous cycle (Fig. 1 and 2). The analysis shows that each ovary contained 8-10 follicles larger than 2mm diameter and that 85% of these were undergoing atresia (6). Medium sized non-atretic follicles were most abundant on day 0 - 5 and from day 9 - 13 of the cycle while large non-atretic follicles (> 10mm diameter) were found on day 4 - 9 and day 13 - 18. The follicle that finally ovulated generally originated from the medium sized class and grew rapidly after regression of the corpus luteum. Occasionally the preovulatory follicle developed from the large follicles found on day 13 - 18.

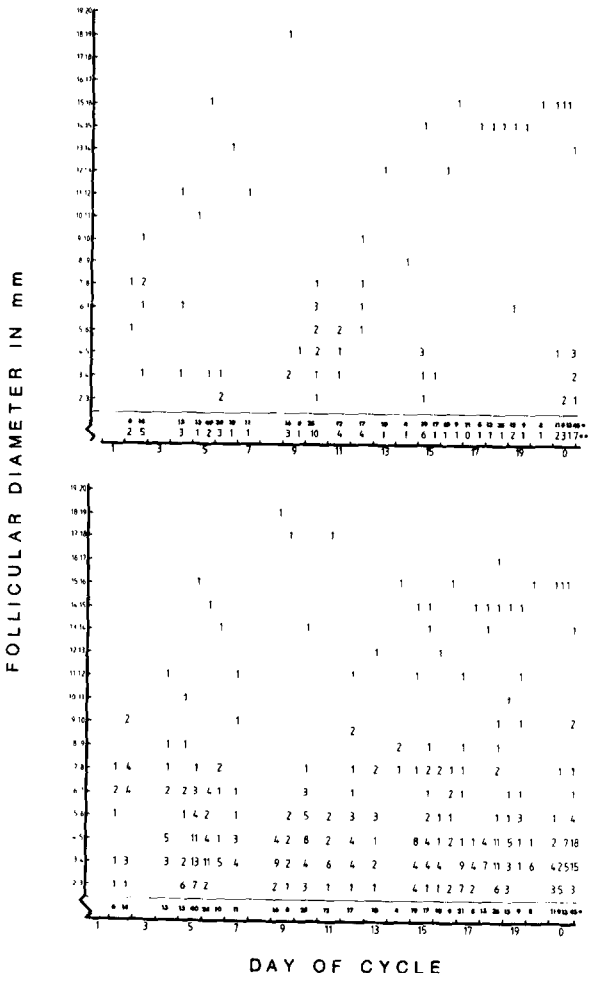

Fig. 1 (Lower diagram) Distribution according to size of all follicles over 2mm diameter in the ovaries of 32 cows slaughtered at random throughout the estrous cycle. The total number of follicles (>2mm diameter) from each cow is shown adjacent to the asterisk*.

Fig. 2 (Upper diagram) Size distribution of the non-atretic follicles (>2mm diameter) from the 32 cows slaughtered during the estrous cycle. *Total number of follicles (>2mm) per cow; ⁑ Number of non-atretic follicles (>2mm) per cow.

Information on bovine folliculogenesis raises the associated question about the state of the oocytes at different cyclic stages and in different follicle classes. This has been analysed by examining the nuclear state of oocytes in the entire follicle population (>2mm diameter) of 11 animals slaughtered at random throughout the estrous cycle. The results presented in Table 1 show that all oocytes in non-atretic follicles, and almost all in follicles undergoing early atresia, remain in the normal germinal vesicle stage. With the progression of atresia the percentage of oocytes showing degenerative changes, generally first observed as a clumping of the chromatin, increases. Resumption of meiosis occurs in the preovulatory follicle just after the LH surge; oocytes in smaller non-atretic follicles are unaffected by the elevated levels of gonadotrophin at estrus.

TABLE 1. Percentages of different classes of bovine follicles and the nuclear stage of their oocytes in normal cycling, untreated and FSH/PMSG treated cows (GV= germinal vesicle; PM= pre-metaphase; MI= meiosis I; Deg.= degenerate).

	Follicular population		% of oocytes at various nuclear stages		
	class	%	GV	PM + MI	Deg.
Untreated 11 cows 222 follicles	1	16.2	100	-	-
	2	18.9	97.6	-	2.4
	3	41.0	94.5	-	5.5
	4	23.9	79.3	1.9	18.8
Treated 4 cows 99 follicles	1	38.4	81.6	18.6	-
	2	31.3	100	-	-
	3	19.3	89.5	-	11.5
	4	11.1	72.7	-	27.3
Key to classes	1 - Non atretic		2 - Slightly atretic		
	3 - Atretic		4 - Severely atretic		

b. Effect of exogenous gonadotrophins on follicle population dynamics

The most detailed study of the short-term action of gonadotrophins on the bovine follicle population has been carried out by Monniaux and colleagues (11). This study shows that PMSG injection significantly increases the mitotic index in preantral and very small antral follicles but is without effect on the larger antral follicle population. Consequently numbers of preantral follicles increase after PMSG but the number of normal antral follicles (>1.7mm diameter) remains unchanged. Since PMSG does not increase the number of antral follicles how is superovulation induced? The answer lies in the capacity of exogenous gonadotrophins to prevent or reverse the process of atresia. A reduction in atresia within those classes of follicles over 1.7mm diameter directly increases the number of follicles able to respond fully to gonadotrophin; follicles under this minimum size are incapable of reaching the preovulatory size in the 4-5 days between gonadotrophin

injection and the LH surge. In calculating the number of responsive follicles it must, however, be stressed that some of the atretic follicles luteinize rather than ovulate after rescue and are therefore of no value in the superovulatory procedure.

Our recent findings strongly support the concept that exogenous gonadotrophin reduces atresia in superovulated animals. Four animals were treated with gonadotrophin and 48h thereafter the entire antral follicle population (3.0mm) was dissected out and analysed by the method of Kruip (6). The results, summarized in Table 1, showed that 70% of follicles in the treated animals were either entirely non-atretic or only lightly atretic as compared with only 35% in untreated animals.

It is perhaps appropriate to examine the extent to which these observations on follicle population dynamics and on gonadotrophin action can explain or improve superovulation procedures. Firstly, the results demonstrate clearly why gonadotrophin injection on day 8-10 elicits the highest ovarian response. It is evident from Fig. 1 and 2 that bovine ovaries at that stage contain the largest number of medium sized antral follicles; the hormonal response experiments show that these classes of follicles respond most favourably to exogenous gonadotrophins. Secondly, it might be possible to increase the number of responsive follicles by exploiting the known stimulatory action of exogenous gonadotrophin on the preantral follicle classes. A protocol of repeated hormone treatments together with the alteration of steroid balances by immunization might be productive in this regard.

An additional important question concerns the long term effect of exogenous gonadotrophin on ovarian function. The conflicting evidence on the effect of repeated hormonal treatments has been clearly documented by Gordon (12). The majority of reports indicate that repeated hormonal stimulation is generally accompanied by a reduction in ovarian responsiveness. These conclusions have received recent support from a large study in which 1192 beef cows were repeatedly superovulated with FSH-P (13). It was concluded from the study that embryo production per collection, analysed on a within group basis, declined with repeated superovulations. Moreover, the decline in embryo production could not be corrected by increasing the amount of FSH-P injected.

We have recently carried out a similar analysis on the effect of repeated superovulations in 68 normal and problem cows treated with PMSG rather than FSH-P (14). Each animal in this study was injected with 2700 iu PMSG at the first treatment; the dose was increased by approximately 200 iu for each treatment thereafter. The effect of repeated treatments on ovulation rate, number of viable embryos recovered, proportion of viable embryos (number of viable embryos over ovulation rate) and proportion of total embryos recovered at each flushing was analysed by ANOVA.

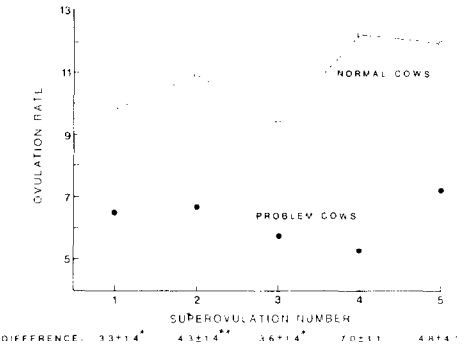

Fig. 3. The effect on ovulation rate of superovulating 68 normal and problem breeder cows at consecutive intervals of 40-60 days with PMSG (14). For details of statistical differences see Fig. 4 below.

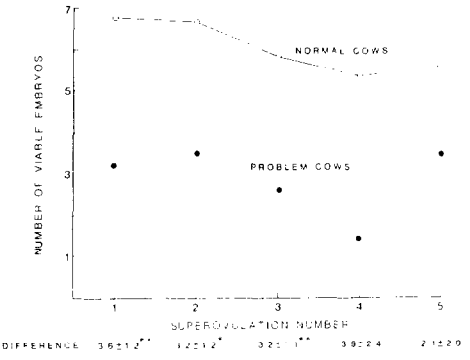

Fig. 4. Mean number of viable embryos recovered from normal and problem breeder cows after repeated superovulations. The DIFFERENCE column shows the means and standard errors of the differences between the two groups of cows. Absence of statistical differences (denoted by asterisks) between groups during the fourth and fifth treatment reflected the small numbers of cows treated at these times.

The findings summarised above (Figs 3 and 4) demonstrate that the variable was maintained at a constant level throughout the experiments. An analysis of the differences in the variable at different treatments failed to reveal any effect of repeated superovulations on either the ovulation rate or on the number of viable embryos recovered. It will be observed that the superiority of normal animals over the problem breeders was evident at the first treatment and maintained thereafter. When the data for each animal was summed over all treatments it was found that the proportion of viable embryos was significantly higher for the normal cows: 87.1% as compared with 75.7%, $P<0.01$.

It is not possible to ascertain from our results the extent to which the gradually increasing dose of PMSG contributed to the consistency of response between the first and fifth hormonal treatment. It is, however, clear that an acceptable response to PMSG can be sustained for at least five treatments. Equally important is the observation that embryo quality does not decline in cows which have been repeatedly superovulated.

GONADOTROPHIN HORMONES AND EMBRYONIC VIABILITY

Superimposed on the problems of inherent ovarian variability are those associated with the quality of embryos produced by superovulation. Results summarized by Newcomb (15) indicate that 55% of embryos recovered from superovulated donors on day 8 show developmental abnormalities. The cause of these abnormalities has generally been ascribed to an unfavourable uterine environment induced by the grossly distorted level of circulating steroids in these animals (16). However, inappropriate interactions between exogenous gonadotrophins and partially differentiated thecal or granulosa cells could equally initiate lesions during maturation which may not be expressed until the morula or blastocyst stage. A clear example of delayed expression of an early abnormality has been reported by Moor and Trounson (17). These workers found that ovine oocytes matured in the presence of inadequate levels of oestrogen underwent fertilization and cleavage but later showed aberrant blastulation patterns. It has been to determine whether similar events occur during superovulation that recent experiments have been carried out in our laboratory (18). The hypothesis under test has been that egg quality is predetermined by an early series of interactions between the exogenous gonadotrophin and follicle cells.

The experiments were carried out using ovine follicles obtained from untreated animals or those treated 36h previously with an equivalent dose of either PMSG or an equine pituitary preparation (FSH-E). After dissection, the follicles were cultured for 24h in the absence of gonadotrophin. Oocytes enclosed by follicle cells were then labelled for 3h in ^{35}S methionine and the radiolabelled proteins in each oocyte separated by SDS gel electrophoresis.

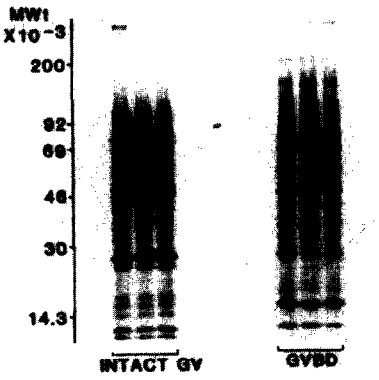

Fig. 5. Comparison of ^{35}S methionine labelled proteins synthesized by non-activated dictyate oocytes (GV) and by maturing oocytes after breakdown of the germinal vesicle (GVBD) and formation of the metaphase plate.

Oocytes obtained from untreated sheep invariably showed the protein profile which characterizes the inactivated germinal vesicle stage of development. Figure 5 shows the protein profile from these control oocytes together with the profile of an oocyte at the metaphase II stage of maturation (GVBD stage). When follicles were obtained from FSH-E stimulated animals approximately 94% of oocytes showed the standard germinal vesicle (GV) pattern of labelled proteins. These results suggest that FSH stimulates follicular development without prematurely activating the associated oocyte. The effect of PMSG was, however, entirely different. A superovulatory dose of 1250 iu PMSG not only stimulated follicular development but also resulted in the premature activation of over 33% of the oocytes.

We have recently extended these observations by carrying out a somewhat similar study using bovine oocytes. Our purpose has been to determine the degree to which premature oocyte activation occurs after gonadotrophin injection in cows; no attempt has been made in these experiments to compare FSH-E with PMSG. Nuclear configuration rather than protein synthesis has been used to assess premature activation in bovine oocytes.

In the first study four animals were injected with gonadotrophin and the cumulus and oocytes examined 48h later (see Table 1). Expansion of the cumulus and resumption of meiosis occurred in 18.4% of the large non-atretic follicles but in none of the atretic classes of follicles. Animals in a second study were injected with 20mg of FSH-E and slaughtered 24h later. Oocytes in the majority of large non-atretic follicles (77.5%) were prematurely activated (Table 2). In the medium sized follicle classes (>6mm diameter) 9% of oocytes showed signs of activation and 19% were degenerate. It is thus evident that premature activation is an important source of loss during superovulation. Some of the activated oocytes will be retained in luteinized follicles while others will be ovulated as aged eggs and will subsequently contribute to the pool of abnormal embryos.

TABLE 2. Nuclear maturation of oocytes in large (>6mm) and small to medium sized bovine follicles collected 24h after FSH-E treatment (20 mg equine pituitary extract).

Size of follicle	No. of follicles	% at each stage of nuclear maturation				
		GV	PM	MI	MII*	Deg.
>6mm	31	19.4	6.5	64.5	6.5	3.2
3 - 6mm	100	82.0	2.0	7.0	0	19.0

* Meiosis II

These different experiments are of interest for two reasons. Firstly they show that exogenous gonadotrophins grossly disturb the nuclear and cytoplasmic function of a significant number of oocytes. Secondly, the results from the experiments on ovine oocytes suggest that

PMSG is much more liable than FSH to disrupt the normal physiological function of the gamete. This latter finding accords well with the practice in many laboratories of using FSH rather than PMSG for superovulations (19).

The chemical properties of the two gonadotrophic preparations provide a probable explanation for the differences in hormonal action on the oocyte. It is, of course, well established that the gonadotrophins share basic similarities. They are composed of structurally similar α-subunits and biologically specific β-subunits. In addition each hormone has a characteristic carbohydrate composition consisting of neutral sugars, hexosamines and a highly variable sialic acid content which determines the half-life of the molecule. Thus, the very low level of sialic acid in LH and the slightly higher content in FSH are reflected in half-lives of 30 min and 110 min for LH and FSH, respectively (20). By contrast, PMSG has an exceptionally high sialic acid content which confers upon it a half-life greatly in excess of that of the pituitary gonadotrophins. In cattle the clearance of PMSG involves a rapid ($t_{\frac{1}{2}}$ = 40-50h) and a slow ($t_{\frac{1}{2}}$ = 118-123h) component (21). The *in vitro* activity of this molecule is further complicated by its dual gonadotrophic activity. Radioreceptor analyses of PMSG suggest an FSH:LH ratio of 1.08 whereas both the amino acid sequence data and the bioassay data (FSH:LH ratio 1:12) are strongly indicative of an LH-like preponderence in the PMSG molecule (21, 22, 23). The slow clearance rate of PMSG coupled with its dominant LH-like activity *in vivo* have the combined disadvantage of exposing stimulated follicles to continuously high LH levels during development. The consequence of this is the premature partial activation of oocytes in those follicles showing the greatest degree of differentiation at the time of hormone injection.

In conclusion, we postulate that the numbers of prematurely stimulated follicles will be substantially reduced by manipulating the FSH and LH levels during superovulation. A reduction in the high LH content of pituitary gonadotrophin preparations should decrease premature activation of oocytes during superovulation. Chemical modifications to the PMSG molecules or a reduction in its half-life by the injection of anti-PMSG serum (24), should equally increase the biological effectiveness of this hormone. Despite these projected improvements availability of viable eggs will still be a major limitation especially if genetic manipulation or nuclear transfer becomes important in animal breeding. These requirements could, however be satisfied by utilizing oocytes from the large pool of ovaries available daily at commercial abattoirs.

OOCYTES, FOLLICLE CELLS AND MATURATION IN VITRO

Oocytes recovered from antral follicles are characterized by their total inability to support even the earliest phases of embryonic life. The acquisition of biological competence in these cells necessitates many structural and biochemical changes which together constitute the process of oocyte maturation. The complexity of this process has

repeatedly been illustrated in the past by the inability to induce the complete range of maturational changes in vitro. Oocytes removed from antral follicles readily resume meiosis in culture, but otherwise remain incompetent and show numerous developmental abnormalities at fertilization or during blastulation (17, 25). It has been to identify the processes by which competence is conferred upon the oocyte and to utilize this information in the development of simple techniques for its induction in vitro that much of our recent work has been undertaken.

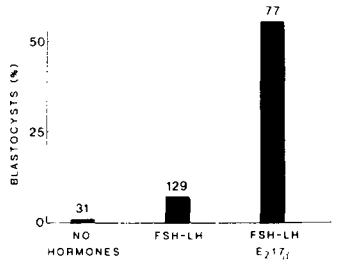

Fig. 6. Effect of difference in hormonal support on the percentage of intrafollicular oocytes that developed into expanded blastocysts. The number of oocytes/group is shown above each histogram (17).

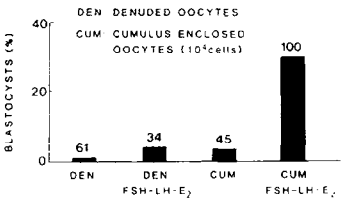

Fig. 7. Effect of cumulus cells and hormones on the percentage of extrafollicular oocytes cultured in a static system that developed into expanded blastocysts. The number of oocytes/group is shown above each histogram.

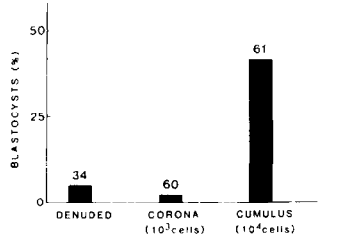

Fig. 8. The effect of cumulus cell numbers on the percentage of extrafollicular oocytes, cultured in a non-static system, that developed into expanded blastocysts. Essential hormones (FSH-LH-E_2 17β) were included in all cultures (26).

The initial experiments were designed to develop a reliable system for the maturation of oocytes within the follicle. The essential features of that study, outlined in Fig. 6-8, were that a high proportion of intrafollicular oocytes acquire full biological competence in vitro provided only that both gonadotrophins and estradiol-17β are included in the medium. Additional experiments demonstrated that the intrafollicular signals elicited by the added hormones originate in the follicle cells and are required by the oocyte during the first 6h of maturation.

The practical requirement for large numbers of mature oocytes has necessitated a re-orientation away from the technically demanding culture of intact follicles. Basic information gained from the intrafollicular system has been used to develop a simple system for the culture of ovine oocytes outside the follicle. The results outlined in Figure 7 show that oocytes denuded of their follicle cells do not acquire developmental competence irrespective of the hormonal support provided. By contrast, cumulus-enclosed oocytes cultured in a normal static system with the appropriate hormone additions develop normally in about 30% of instances (27). Two recent findings have provided the basis for a highly significant increase in the percentage of extrafollicular oocytes that mature in vitro. Our first observation was that gentle agitation of the cultures prevents attachment and consequent premature luteinization of the granulosa cells; maintenance of the correct state of somatic cell differentiation during culture markedly increases the percentage of oocytes undergoing maturation (Fig. 8). A further improvement was effected by adding supplementary granulosa cells to the culture system. The addition of 5×10^6 cells/ml increased to 37% the number of blastocysts from corona enclosed oocytes and to 55% those formed from cumulus enclosed oocytes (26). The 55% of blastocysts formed from oocytes matured in vitro compares favourably with 65% formed when oocytes matured normally in vivo were transferred to host recipients. Our results suggest that this simple procedure will, with minor modifications, be suitable for the maturation of oocytes from many species including the cow.

SUMMARY AND CONCLUSIONS

The objective of obtaining a large but predictable number of viable embryos by superovulation depends both upon the presence of responsive follicles and the administration of appropriate hormones. We conclude that neither of these key determinants have yet been optimized.

An analysis of folliculogenesis in untreated and superovulated cows demonstrates that exogenous gonadotrophin stimulates mitotic activity in preantral follicles and reduces atresia in antral follicles. It is, however, on the number, size distribution and condition of the antral follicle classes that the degree of stimulation directly depends. From present results it seems improbable that the intraovarian control mechanisms can be sufficiently controlled to eliminate inherent differences in follicle dynamics and thereby overcome this primary source of variability during superovulation. Significant improvements within these primary physiological constraints can, nevertheless, be expected from new work on the nature of the administered hormones and their mode of application. Results show that both the number and viability of embryos are directly affected by the hormonal schedules administered.

Future demands for large numbers of eggs for twinning, nuclear transfer and genetic manipulation will be met by utilizing oocytes from the large number of ovaries available at commercial abattoirs. Full physiological maturation is induced by the provision of obligatory

somatic signals and the maintenance of essential cellular interactions during the culture of these oocytes in an extrafollicular environment.

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