BRIEF COMMUNICATION

THE STORAGE OF FOWL SPERMATOOA

I. PRELIMINARY RESULTS WITH NEW DILUENTS

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Summary. Semen obtained from Rhode Island Red males was stored for 24 hr at 2 to 5°C in two diluents containing skimmed milk. The fertilizing ability of the spermatozoa was tested afterwards in White Leghorn and Rhode Island Red hens and the results showed that fowl spermatozoa could be stored for 24 hr without considerable loss of their fertilizing capacity.

Polge (1955) stored fowl semen in a glucose-citrate solution with either milk or thin egg albumen. Promising fertility results (50%) were obtained after 24 hr storage. Schlindler, Weinstein, Moses & Gabriel (1955) used synthetic and natural media as diluents and fertility results were poor after 24 hr storage. Lake (1960) used a diluent, calculated to have an osmotic pressure and ionic composition similar to lipid- and protein-free seminal plasma, to which was added fructose. Semen, free of transparent fluid, was stored for 24 hr and 64% fertility was obtained.

Wilcox & Shorb (1958), Wilcox & Shaffner (1958), Wilcox (1958, 1959a, b, 1960), Wilcox & Wilson (1961) and Wilcox & Clark (1962) used various modifications of a phosphate buffer with antibiotics and diluted the semen in the ratio of 1 part semen to 9 parts diluent for storage. Afterwards the mixture was centrifuged and the spermatozoa were resuspended in buffer plus fructose, to the original semen volume, before insemination. Sixty to 70% fertile eggs were obtained during 1 week after insemination when the semen had been stored for 48 hr at 2°C. Blackwood & Harris (1960) and Harris, Wilcox & Shaffner (1961) used a metabolic inhibitor (2-ethyl-5-methyl-benzimidazole) to prolong the life of cock spermatozoa during storage for 5 days, and 75% fertility was obtained thereafter with washed spermatozoa. Sacki (1960), Wales & White (1961) and Yamane, Tsukunaga & Takahashi (1962) suggested that hypertonic diluents were beneficial to the survival of fowl spermatozoa *in vitro*. Subsequently Hobbs & Harris (1963a, b) and Harris & Hobbs (1964) obtained promising results with hypertonic citrate and bicarbonate diluents, although the osmotic pressure of the diluents was different. The object of the

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present study was to investigate new diluents for the storage of fowl semen for 24 hr at 2 to 5°C. The experiments were conducted from November 1965 to September 1966.

Semen from 8-month-old Rhode Island Red (R.I.R.) males (Commercial, Hubbard Autosex strain) of proven fertility was used throughout these experiments. Two types of hens, White Leghorns (Commercial, Stones strain) and R.I.R. (Hubbard Autosex), in their 1st laying year were chosen at random for making tests of fertility. Both males and females were kept in individual cages.

Lorenz (1964) suggested certain procedures for the best technique of collecting, diluting and storing fowl semen *in vitro*. In the present study semen was collected from fifteen males by a two-man technique (Burrows & Quinn, 1935) and care was taken to minimize contamination with transparent fluid. The pooled semen was transferred to tubes in a water bath (37°C) and diluted as quickly as possible, usually within 15 min, in either of two diluents (Table 1). Semen was pipetted into the pre-warmed diluent in stoppered tubes of 10 ml

TABLE 1
COMPOSITION OF DILUENTS*

<i>Stock solutions</i>	<i>Diluent 1 (ml)</i>	<i>Diluent 2 (ml)</i>
Skimmed milk	50	20
Sodium glutamate (0.1 g/ml)	24	20
Sodium citrate (dihydrate) (0.1 g/ml)	7.7	7.7
Citric acid (0.1 g/ml)	0.3	1.3
D-Glucose (0.1 g/ml)	17	20
Egg albumin	—	10
Ethanol	0.2	0.2
Water (glass-distilled)	0.8	20.8

* Each diluent contained 200 mg dihydrostreptomycin sulphate and 40 mg oxytetracycline hydrochloride/100 ml.

capacity (1 volume semen to 1 volume diluent) and gently mixed with a stirring rod. The combined volume of semen and diluent never exceeded 3 ml. Some tubes were stoppered with cotton wool and others with rubber bungs. The diluted semen was placed in a refrigerator for storage at 2 to 5°C.

In preparing diluent 1, the skimmed milk was heated to 90 to 95°C (Van Wambeke, 1964) for 10 min, cooled and filtered before adding to the mixture of the appropriate proportions of citrate, citric acid, glutamate and glucose in an Erlenmeyer flask. The oxytetracycline was dissolved in 0.2 ml ethanol and 0.8 ml glass-distilled water and then placed in the diluent which was completed by adding 200 mg streptomycin. For diluent 2, the albumin of freshly laid eggs was homogenized and pipetted into an Erlenmeyer flask after which the glucose, glutamate, citrate and citric acid were added. The skimmed-milk, glass-distilled water and antibiotics were added to give the final volume of 100 ml.

The freezing point depression, Δ , of the diluents was determined with an Astell cryoscope and the pH with a pH meter 22 (Radiometer, Copenhagen).

The hens were inseminated as soon as possible with 0.10 to 0.12 ml of cool semen after it had been taken from storage. Four groups (ten to fifteen birds each) of White Leghorns and two groups of R.I.R. hens selected from thirty-one birds were inseminated on different days. The semen was deposited deep in the vagina, and in a few cases the utero-vaginal junction was penetrated so that the spermatozoa entered directly into the shell gland. Inseminations were performed in the afternoon and hens with a hard-shelled egg were not inseminated. Fertility results were based on the number of fertile eggs laid from the 2nd to the 8th day inclusive after insemination. The eggs were broken out after 3 to 5 days incubation and embryonic development was observed. A hen was classified as fertile when she had laid at least one fertile egg during the 7-day period.

The results, summarized in Tables 2 and 3, show that fowl spermatozoa can be stored for 24 hr without considerable loss of fertility. There was a suggestion of a slight difference between the fertility results obtained from the two strains of hens especially when diluent 1 was used. However, further investigation of this possibility is required.

TABLE 2
PERCENTAGE FERTILE EGGS LAID DURING DAYS 2 TO 6 INCLUSIVE BY HENS INSEMINATED
WITH SEMEN STORED IN DILUENT 1 FOR 24 HR AT 2 TO 5°C

<i>Breed of hen and type of stopper in storage tube</i>	<i>Group</i>	<i>All hens</i>			<i>Fertile hens</i>		
		<i>No. hens</i>	<i>No. eggs laid</i>	<i>Percentage fertile eggs</i>	<i>No. hens</i>	<i>No. eggs laid</i>	<i>Percentage fertile eggs</i>
White Leghorn: rubber bung	1	14	66	76	12	58	86
	2	10	36	94	10	36	94
	3	11	51	71	9	45	80
	4	14	76	92	14	76	92
Total	—	49	229	83	45	215	88
Rhode Island Red: cotton-wool plug	2	13	68	99	13	68	99
	2	14	78	91	14	78	91
Total	—	27	146	95	27	146	95

A few W.L. hens failed to become fertilized despite successive inseminations with stored semen. However, when fresh spermatozoa were deposited into their vagina, these hens produced normal fertile eggs. In contrast, the R.I.R. hens were all fertilized. Whether this was due to strain differences or to the insemination technique, remains to be investigated. Insemination procedure appeared to be critical because mid-vaginal inseminations resulted in more infertile W.L. hens than did depositing the semen close to or, perhaps, through the utero-vaginal junction. The R.I.R. hens, which are heavier birds and have larger reproductive organs, were less influenced by the insemination technique; furthermore their rate of lay remained undisturbed. A few W.L. hens stopped laying for different periods, probably on account of the forcible penetration of the utero-vaginal junction (Bohr, Lake, Lorenz, Ogasawara & Krzanowska, 1965).

The pH of the diluents was 6.75 and 6.1 for diluents 1 and 2 respectively. When 0.5 ml of 0.1 M HCl was added to 10 ml of both diluents 1 and 2, the pH was lowered 0.3 and 0.2 units respectively. The pH of the semen in diluent 1 varied from 7.1 (0 hr) to 6.65 (24 hr) during storage in tubes with cotton-wool plugs. On the other hand, semen diluted with diluent 2 had an initial pH of 6.5 and after 24 hr the pH was 6.45 and 6.25 in tubes with rubber bungs and cotton-wool plugs respectively. These observations agree with the findings of Wilcox (1959a) who showed that fertility was not affected between wide pH ranges (6.5 to 8). However, Wilcox used different conditions of storage. Both diluents were hypertonic to seminal plasma ($\Delta -1.1^\circ$ for 1 and -0.98° for 2) and thus, like Yamane *et al.* (1962), Hobbs & Harris (1963a, b) and Harris & Hobbs (1964), it has been found that spermatozoa can retain their fertilizing capacity in hypertonic media.

TABLE 3
PERCENTAGE FERTILE EGGS LAID DURING DAYS 2 TO 6 INCLUSIVE BY HENS INSEMINATED
WITH SEMEN STORED IN DILUENT 2 FOR 24 HR AT 2 TO 5° C

Breed of hen and type of stopper in storage tube	Group	All hens			Fertile hens		
		No. hens	No. eggs laid	Percentage fertile eggs	No. hens	No. eggs laid	Percentage fertile eggs
White Leghorn: cotton-wool plug	1	14	64	94	14	64	94
	2	10	46	98	10	46	98
Total	—	24	110	95	24	110	95
White Leghorn: rubber bung	1	14	68	94	14	68	94
	2	10	45	93	10	45	93
	3	10	51	82	9	45	93
	4	15	71	89	15	71	89
	1	14	77	92	14	77	92
	2	9	41	93	9	41	93
Total	—	72	353	92	71	347	92
Rhode Island Red: rubber bung	1	16	83	89	16	83	89
	1 + 2	28	144	93	28	144	93
	1	15	66	98	15	66	98
Total	—	59	293	93	59	293	93

Further research is planned to investigate the precise interrelationship between pH and osmotic balance. The dilution ratio and storage temperature were chosen arbitrarily. It would be of practical interest to know how much the dilution ratio and storage time could be extended without considerably reducing the fertility. The hens were inseminated with 0.10 to 0.12 ml of stored semen which is equivalent to 0.05 ml of undiluted semen. Insemination with a double dose produced no better fertility, therefore an adequate number of spermatozoa remained viable after 24 hr storage.

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