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An attempt to improve the fertility of stored fowl semen with certain additives in a basic diluent

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Summary. The following substances were added to a basic diluent for fowl semen either separately or in combination : caproic acid, formaldehyde, acetyl carnitine, adenine, inosine, sodium pyruvate, succinic acid, dithiothreitol, sodium citrate and zinc, chloride, bicarbonate and phosphate. No significant improvement in the fertility of semen stored at 5 °C for either 24 or 48 hrs was obtained over that produced with the basic diluent. Dithiothreitol and sodium citrate caused a marked lowering of fertility when present in the diluent.

Introduction.

Reproduction in domestic avian species by artificial insemination presents a unique problem, because after holding semen *in vitro* sufficient spermatozoa must be in a condition to be able to survive in the oviduct for a further period, of at least a week, in order to fertilize all the eggs which may be ovulated daily by the female. About 100 million good quality spermatozoa must be inseminated to achieve an economic fertility level under such conditions (Lake and Stewart, 1978). It is thus very important to minimize the loss of spermatozoa *in vitro*.

With respect to influencing the number of good quality spermatozoa, an appreciation of the anatomy of the bird is important during the collection of semen. The presence of a cloaca increases likelihood of obtaining semen contaminated with faeces and urine, which can cause damage to spermatozoa. Transparent fluid emanating from lymphatic folds (Lake, 1981) can be collected with semen, the amount depending upon the method of collection, the type and excitability of the donor bird. Transparent fluid can also damage spermatozoa (Lake, 1981) but in any case reduces the initial density of spermatozoa in collected semen. This becomes an additional factor in determining the dilution rate for semen and the calculation of its dosage to achieve optimum fertility from a hen after insemination (Lake and Stewart, 1978).

Good fertility can be obtained with uncontaminated fowl semen stored above 0 °C for up to 24 hr (Lake and Ravie, 1979 ; Van Wambeke, 1967, 1972). It should be possible to improve storage techniques and extend the storage period by manipulating the metabolism of spermatozoa *in vitro*. Recently, MacPherson, Fiser and Reinhart (1977) reported a beneficial effect of caproic acid on the retention of fertilizing capacity of stored fowl spermatozoa. Fiser and MacPherson (1978), Fiser, MacPherson and Rein-

hart (1978) and Fiser *et al.* (1980) studied the effect of sodium pentobarbital in diluents with fowl and turkey spermatozoa ; it is believed to affect mitochondrial metabolism. No conclusive evidence was obtained that fertility of stored turkey semen after 6 and 24 h storage was improved. There was some indication of an improvement with fowl semen, although fertility results after 48 h were in disagreement with those after 24 h storage. Esashi, Suzuki and Sahashi (1969) added inosine and adenine to a fowl semen diluent, inosine having been shown to affect the liver pyruvate system, and claimed that inosine alone caused an improvement in fertility.

This paper describes the effects on fertility of storing fowl semen at 5 °C for 24 or 48 h in a basic diluent with or without several ions or compounds alone or in combination that have been shown from general cell studies to either regulate features of cell metabolism or directly affect cell membrane integrity.

Materials and methods.

Animals. — A population of 150 hens and 20 males were used for testing the fertility of stored semen. They were of a commercial layer-type. All birds were maintained on a commercial breeder's ration and fed *ad libitum*. They were given 14 h light/24 h.

Semen treatment. — Semen was collected, diluted in several diluents and stored at 5 °C according to the method described by Lake and Ravie (1979). The composition of

TABLE 1

Composition of the diluents used for storing fowl semen for 24 or 48 h at 5 °C

Compound	Diluents					
	7.10	DTT	IN	AC	NC	GM
Sodium glutamate. H_2O	0.76	0.76	0.76	0.55	—	0.288
Tri-potassium citrate. H_2O	0.064	0.064	0.064	0.064	0.064	0.064
Magnesium acetate. 4 H_2O	0.04	0.04	0.04	0.04	0.04	0.04
Sodium acetate (anhydrous)	—	—	—	0.073	0.073	0.099
Glucose	0.3	0.3	0.3	0.18	0.3	0.3
1 N-NaOH	2.8 ml	2.8 ml	2.8 ml	2.8 ml	2.8 ml	2.8 ml
BES[+]	1.525	1.525	1.525	1.525	1.525	1.525
Zn acetate. 2 H_2O	—	—	—	—	—	0.002 4
Inosine	—	—	0.057	—	—	0.057
Adenine	—	—	—	—	—	0.004 5
Sodium pyruvate	—	—	—	—	—	0.001 84
Succinic acid	—	—	—	—	—	0.012
Sodium chloride	—	—	—	—	0.052	0.037
Di-sodium hydrogen phosphate (anhydrous)	—	—	—	0.068	0.063 5	0.044 5
Sodium bicarbonate	—	—	—	—	0.053	0.053
Sodium citrate. 2 H_2O	—	—	—	—	0.1	—
Acetyl carnitine. HCl[*]	—	—	—	0.005 99	—	—
DL-Dithiothreitol[*]	—	0.038 6	—	—	—	—
pH	7.1	7.1	7.18	7.1	7.2	7.1
Δ (mosmol/kg H_2O)	406	414	413	407	390	417

[*] Sigma chemical Co. Ltd.

[+] NN-Bis (2 hydroxyethyl)-2-aminoethane sulphonic acid.

Values are in g, unless otherwise stated, dissolved and made up to 50 ml with distilled water.

a basic (7.1) and other diluents containing several different substances, alone or in combination, is given in table 1. The following additional modifications of the basic diluent were also made and tested :

a) Different amounts of n-caproic acid (99-100 p. 100, Sigma Chemical Co. Ltd) were added such that 2-fold dilutions of semen had the following final concentrations — (0.02, 0.045, 0.06, 0.12 and 0.5 p. 100 v/v).

b) Different amounts of formaldehyde (37-40 p. 100 w/v, AR grade, Hopkins & Williams) were added such that a 2-fold dilution of semen had the following final concentrations (0.006, 0.012 and 0.018 p. 100 v/v).

Samples of diluted semen were stored using diluents 7.1, GM, AC and IN for 48 h and were shaken during the entire period. In these cases, oxygen was found to be present continouusly in the medium. Diluted semen was stored for 24 h in diluents DTT, NC (table 1) and those modifications of the basic containing caproic acid and formaldehyde and were held in standing tubes where free oxygen was found to be absent from the medium for most of the storage period (G. J. H. Wishart, personal communication).

Insemination procedure and fertility tests. — Groups of hens were used sequentially to test the diluents. The basic diluent (7.1, table 1) was used for the freshly diluted control included with each batch of tests. Hens were inseminated with 0.06 ml diluted semen containing about 150×10^6 spermatozoa because the fresh semen contained between 4.5 and 5.5×10^6 spermatozoa/c.m.m. and the dilution ratio was 1 : 1. The diluted semen samples were held at room temperature (15 °C) for no more than 30 min after taking from the cold water storage bath. This time included the time taken to complete the inseminations. Eggs were collected for 15 d after each insemination, set in an incubator and candled after 6-7 d incubation to determine fertility. Any cracked unincubated eggs or eggs showing doubtful embryonic developments on incubation, were broken open and the blastoderms inspected for evidence of fertilization. The percentage of fertile eggs laid by each hen during days 2 to 8 and 9 to 15 after insemination and the mean percentages for these periods for each treatment group were calculated. Percentage data were transformed to arc sine values for analyses of variance. The pooled error mean square was used to test the level of significance of the differences between treatments by Student's t-test.

Results and discussion.

Fertility was depressed with semen stored in the basic diluent at 5 °C for 48 h (B, fig. 1 and 2) compared with after 24 h (2, fig. 1 and 2). However, the levels of fertility obtained with semen stored for 48 h in the basic diluent, with or without acetyl carnitine or inosine, were encouraging (B, D, E, fig. 1 and 2) and indicated that these diluents would allow a breeder more flexibility in breeding by artificial insemination. For example, the breeder could transport semen over considerable distances after collection from the male.

An analysis of the data in figures 1 and 2 showed that when comparing within the 24 or 48 storage periods there was no significant difference in fertility between the results obtained with semen stored in the basic diluent and that supplemented with various combinations of substances expected to stimulate or influence the integrity of

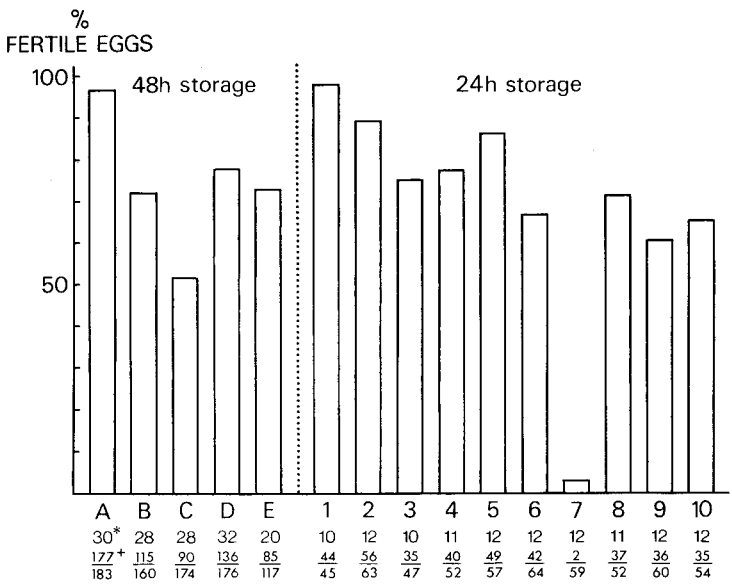

FIG. 1. — *The numbers of fertile eggs produced during days 2-8 inclusive after single inseminations of semen stored for 24 or 48 h at 5 °C in diluents of varying composition* : A. Basic diluent (7.1) ; freshly inseminated control. B. Basic diluent (7.1). C. Basic diluent containing several additional ions or compounds associated generally with stimulating aspects of cell metabolism, e.g. zinc, chloride, phosphate, bicarbonate, pyruvate, succinate, inosine and adenine (GM). D. Basic diluent plus acetyl carnitine (AC). E. Basic diluent plus inosine (IN). 1 : Basic diluent (7.1) ; freshly inseminated control. 2 : Basic diluent (7.1). 3, 4, 5, 6, 7 : Basic diluent containing respectively (final concentration). 0.02, 0.045, 0.06, 0.12, 0.5 p. 100 caproic acid. 8, 9, 10 : Basic diluent containing respectively (final concentration). 0.006, 0.012, 0.018 formaldehyde. * N° of hens ; + N° of fertile eggs/total eggs laid during days 2 to 8 inclusive.

cells in various respects. The basic diluent had already been shown to be suitable for storing fowl semen for 24 h at 5 °C (Lake and Ravie, 1979).

Effect of various concentrations of caproic acid. — The fertility obtained with semen stored for 24 h in the basic diluent was not different from that obtained when the fluid contained caproic acid in all amounts except 0.5 p. 100 (v/v) (3-7, 2, fig. 1 and 2). The latter resulted in poor fertility and was considered to be due to the compound creating a pH lower than 5.5 in the medium. MacPherson *et al.* (1977) claimed an improvement in fertility with the addition of caproic acid to a sodium citrate-citric acid-glycine based diluent using final concentrations in semen of 0.0025, 0.0033 and 0.005 p. 100 (v/v). However, compared to their controls without caproic acid, the fertility obtained, using 0.005 p. 100 caproic acid and a semen dosage equivalent to 0.05 ml original semen, was raised from about 12 p. 100 to only 25 p. 100 after 28 h and from about 55 p. 100 to only 70 p. 100 after 6 h storage. The expressed fertility appeared to be the average for the whole of the 3-wk period post-AI. No fertility figures were given for a freshly diluted aliquot of semen for comparison. The sodium citrate diluent tested in the present work markedly lowered the fertility of semen stored for 24 h at 5 °C (38.5 p. 100, days 2 to 8 and 4.2 p. 100 fertile eggs, days 9 to 15) and so it is suggested that the results of Mac-

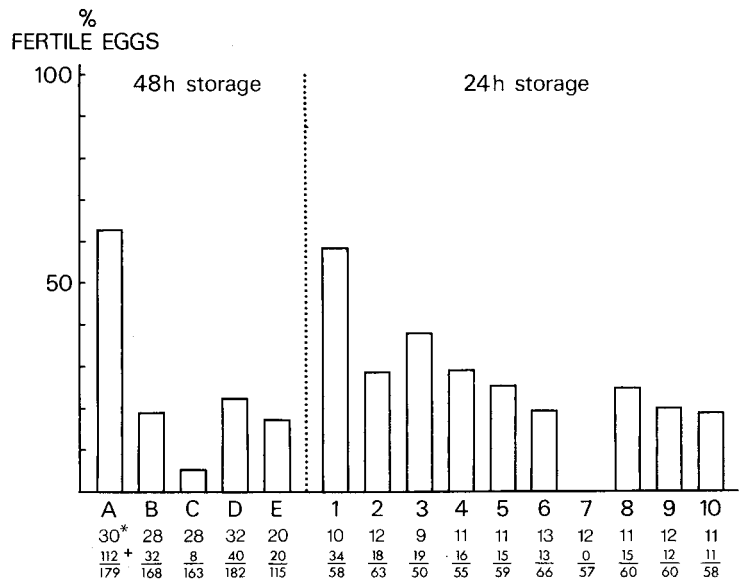

FIG. 2. — *Details the same as for figure* 1, *except that they apply to eggs laid during days* 9 *to* 15 *after the single inseminations.*

Pherson *et al.* (1977) demonstrated merely a stimulation of spermatozoa otherwise inhibited by the sodium citrate solution. It has been shown that sodium citrate tends to reduce the motility and/or metabolism of mammalian (Salisbury, Fuller and Willett, 1941) and avian (Bogdonoff and Shaffner, 1954 ; Auger and Wilcox, 1964) spermatozoa. Shannon (1962) showed that caproic acid enhanced the viability of stored bull spermatozoa compared with other volatile fatty acids. But, in this case, caproic acid was also added to a basic diluent containing much sodium citrate. He was unable to demonstrate conclusively how caproic acid benefited bull spermatozoa.

Effect of various concentrations of formaldehyde. — Formaldehyde at the concentrations used in the present study neither harmed nor improved significantly the fertility of fowl semen stored for 24 h (8-10, 2, fig. 1 and 2). Dott, Moor and Polge (1976) observed normal fertility with freshly diluted boar and ram semen using formaldehyde at a final concentration of 0.012 5 p. 100 in the diluent. They suggested that since it had a stabilizing effect on the membranes of spermatozoa it may be beneficial in minimizing damage during freezing. From the present work, there was no indication that it enhanced the viability of fowl spermatozoa during storage at 5 °C.

Effect of acetyl carnitine. — This compound neither harmed nor enhanced the viability of spermatozoa during 48 h storage (*cf.* B and D, fig. 1 and 2). It is a natural nitrogenous constituent of fowl seminal plasma produced by the lining cells of the male reproductive tract and the amount in the diluent was equivalent to that found naturally (10.55 p. 100, G. J. H. Wishart, personal communication). It is not known whether it penetrates the membrane of the mature spermatozoa to participate in lipid and car-

bohydrate metabolism. However, it was interesting to observe that the fertility of some batches of stored semen was remarkably good and may have reflected a variation in the degree of oxygenation in tubes during shaking for 48 h, thus influencing oxidative metabolism in the presence of acetyl carnitine. This remains to be investigated.

Effect of supplementation of the basic diluent with ions or compounds generally associated with the stimulation of aspects of cell metabolism. — The complex GM solution included several ions or compounds which have been considered in different circumstances as essential for maintaining various aspects of cell metabolism and integrity. Inosine, a nucleoside, and adenine, a purine base, were included since the combination has been shown to maintain ATP levels and the morphology of red blood corpuscules stored at 4 °C (Nakao *et al.*, 1960 ; Nakao, Ozaki and Nagano, 1968) albeit in citrate-glucose solutions. Also, Esashi, Suzuki and Sahashi (1969) reported that inosine alone when added to Ringer's solution improved, but inosine plus adenine lowered, the fertility of stored fowl semen. However, no details of the storage time or temperature were given. The results of the present work showed that the GM solution significantly lowered fertility of stored semen ($p < 0.05$ compared with the basic diluent and $P < 0.001$ compared with the freshly diluted control) (*cf.* C and A, B, fig. 1 and 2). A precise explanation cannot be given but the motility of spermatozoa after storage was very vigorous which may indicate that metabolism was stimulated unduly thus causing a premature loss of cell viability during storage. Contrary to the findings of Esashi *et al.* (1969) no significant improvement in fertility was observed with the use of inosine alone when semen was stored for 48 h at 5 °C (*c. f.* E and B, fig. 1 and 2).

Effect of DTT. — Spermatozoa stored for 24 h at 5 °C in the presence of Dithiothreitol (DTT) produced very low fertility (17.3 p. 100, days 2 to 8 and zero, days 9 to 15 after insemination). No fertility resulted after 48 h storage. Freshly diluted semen containing DTT showed the usual high level of fertility, in this case 96 p. 100, days 2 to 8 and 40.8 p. 100, days 9 to 15 after insemination. The precise reason for the adverse effect of DTT is unknown. It prevents oxidation of -SH groups and consequently may act on membrane function. However, it stimulates glycolysis and lactate production in the isolated rat diaphragm (Haugaard, Smith and Haugaard, 1972) and it is interesting to speculate whether DTT acted similarly on spermatozoa to adversely affect retention of fertilizing capacity. Lake and Ravie (1979) showed that the fertility of fowl semen after storage could be improved by preventing excessive acidification in a diluent. However, it is not known whether acid accumulated in the medium under the conditions of the present work.

In conclusion, the fertility of fowl spermatozoa stored for 24 h or 48 h was not improved by adding ingredients to a basic diluent, already known to yield satisfactory results for 24 h storage (Lake and Ravie, 1979). From general knowledge of cell function, the chosen additives were expected to stimulate or modify metabolism of cells. It is suggested that since mature spermatozoa are incapable of biochemical synthesis, the main aim when storing semen above 0 °C is to prevent them from degenerating and expiring prematurely. One necessity is to provide a type of storage environment which minimizes the development of noxious conditions caused by the accumulation of by-

products of the continuous metabolism of the spermatozoa. For further progress, amongst other things, a more direct knowledge of the factors influencing catabolism of spermatozoa may make possible more constructive attempts to direct the course of metabolism so that the rate of degenerative changes may be slowed.

The temperature of storage, composition of diluents and gaseous environment are also likely to be important tools in the pursuance of this aim.

Deep-freezing offers the only technique of storage whereby the inherent life span of the spermatozoon may actually be increased in a state of suspended animation.

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Résumé. Dans le but d'essayer d'améliorer le pouvoir fécondant de sperme de coq conservé *in vitro*, sans congélation, différents additifs ont été ajoutés à un dilueur de base, seuls ou en combinaison : acide caproïque, formaldéhyde, acétyl-carnitine, adénine, inosine, pyruvate de sodium, acide succinique, dithiothréitol, citrate de sodium et zinc, chlorure, bicarbonate et phosphate. Mais, aucune amélioration de fécondance n'a été constatée par rapport au dilueur de base, le sperme dilué ayant été conservé à 5 °C pendant 24 ou 48 h. Au contraire, le dithiothréitol et le citrate de sodium ont provoqué une diminution marquée de la fécondance.

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