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## Long-term Storage of Bull Semen Frozen at Very Low Temperatures ( $-79^{\circ}\text{C}.$ ).

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### *Introduction.*

The development of suitable conditions for the storage of semen has done much to promote the extension of artificial insemination in cattle breeding. In routine practice at the present time bull semen is generally diluted with media containing egg-yolk and stored at temperatures between  $2^{\circ}$  and  $5^{\circ}\text{C}.$  Under these conditions satisfactory fertilizing capacity of the spermatozoa can be maintained for several days.

The purpose of this communication is to give an account of entirely new experiments which open up the possibility of storing semen for very prolonged periods at temperatures far below those hitherto considered to be optimal, or even compatible with the survival of the spermatozoa.

The importance of temperature as a major factor influencing the survival of spermatozoa *in vitro* has long been recognised. It is generally accepted that the effect of lowering temperature is to reduce the motility and metabolism of the spermatozoa and thereby to prolong their survival, but the application of this concept has hitherto been limited to temperatures above zero by the fact that freezing of the semen is fatal to the spermatozoa. In the work to be described we have been able, by avoiding the injurious effects of solidification of the semen, to carry this concept to its logical extreme and to offer strong support to

the idea that if living cells can be cooled to temperatures low enough to arrest biochemical activity and subsequently rewarmed without damage it should be possible to preserve the cells at these low temperatures almost indefinitely.

There are numerous observations in the literature showing that bacteria and certain other small living cells may resume normal function on thawing after exposure to temperatures approaching absolute zero (*Luyet and Gehenio, 1940*). By contrast, there was little information until recently as to the effect of extreme temperatures on spermatozoa and, in general, temperatures causing the solidification of semen have been found very harmful to spermatozoa, although human semen is exceptional (*Parkes, 1945*).

In the last three years, however, remarkable new developments have been made possible by the discovery that glycerol and some related compounds have the property of protecting spermatozoa of several species when frozen at  $-79^{\circ}\text{C}$ ., the temperature of solid  $\text{CO}_2$  (*Polge, Smith and Parkes, 1949*).

*The preservation of motility of spermatozoa after freezing in the presence of glycerol.*

Initial experiments were carried out on avian semen. Fowl semen undiluted or in Ringer's solution showed no motile spermatozoa after freezing in small ampoules. By contrast, if ampoules of semen containing 15 % glycerol were plunged into a freezing mixture of solid  $\text{CO}_2$  and alcohol at  $-79^{\circ}\text{C}$ ., the specimen, when thawed, would show almost complete recovery of the spermatozoa and, as far as motility was concerned, it was indistinguishable from unfrozen semen (*Polge, Smith and Parkes, 1949*). The duration of freezing was relatively unimportant, as samples thawed out after one year at  $-79^{\circ}\text{C}$ ., showed quite a high proportion of motile spermatozoa (see *Parkes, 1950*).

Later, this work was extended to mammalian spermatozoa. Bull or goat semen diluted to contain 15 % glycerol and frozen fairly rapidly to  $-79^{\circ}\text{C}$ ., by the method successful with fowl semen, showed only a negligible revival of spermatozoa on thawing. If, on the other hand, the glycerol-treated semen was cooled slowly to  $-79^{\circ}\text{C}$ ., the sample could be revived to show a considerable degree of motility. The best revival rates from

bull semen, of the order of 80 %, were obtained when the semen in 15 % glycerol was frozen over the space of about 45 mins. from 0° C. to -79° C., and subsequently thawed in a water bath at 40° C. (Smith and Polge, 1950).

*Fertilizing capacity of frozen bull semen.*

The discovery of the protective action of glycerol together with the method of slow cooling enabled experiments on the fertilizing capacity of frozen bull semen to be undertaken.

In the first experiments, comparisons were made between normal semen, semen treated with glycerol, and semen treated with glycerol and subsequently frozen. The procedure adopted was to split a single semen sample into three parts. One part was diluted at the rate of 1:1 with citrate-yolk buffer (3.92 % sodium citrate, pH 6.7 plus 50 % egg-yolk) and the other two parts were similarly diluted with yolk-buffer containing 30 % glycerol so that the final concentration of glycerol in the semen was 15 %. All dilutions were carried out with the semen and diluent at about 30° C. One of the samples containing glycerol was then frozen slowly by placing ampoules of semen inside a small vacuum flask containing alcohol and immersing this in a larger freezing bath at -79° C. The temperature in the small vacuum flask fell slowly overnight and generally reached -70° C. after about 20 hours cooling. Although the rate of freezing by this method was considerably slower than the rate mentioned above, the revival of spermatozoa appeared to be satisfactory. The day after freezing, ampoules were removed from the cooling vessel and thawed in a water bath at 40° C. The semen was then used for routine insemination, as also were the two control samples which had not been frozen but had been kept in a refrigerator at 5° C. during the time the experimental sample was being frozen. About 20 cows were inseminated with semen which had been treated in each of the three ways described, and those not returning to oestrus within 6 weeks following insemination were examined for pregnancy by rectal palpation. The result of this experiment gave a pregnancy rate in the three groups of 50 %, 76 % and 0 % respectively for untreated semen, semen treated with 15 % glycerol and semen treated with 15 % glycerol and then frozen and

thawed. The pregnancy rate for the normal semen was rather lower than might have been expected. By contrast, results obtained with the semen containing glycerol were surprisingly good, showing clearly that a glycerol concentration as high as 15 % did not diminish the fertilizing capacity of the spermatozoa. The entirely negative results obtained with frozen semen were discouraging and when experiments were resumed, therefore, attention was devoted to improving the conditions of freezing.

Firstly, it was noted that the quick addition of 15 % glycerol to semen at 30° C. caused a marked reduction in the motility of the spermatozoa. Some spermatozoa appeared to be quite immobilised while others showed distinct kinking of their tails. This effect seemed to indicate a sensitivity to osmotic shock which might be reduced by adding the glycerol either more slowly or at a lower temperature. Nevertheless, even in semen showing good motility after the slow addition of glycerol, revivals after freezing were not much improved.

It was then discovered that samples of semen diluted with glycerol at about 5° C. and allowed to equilibrate overnight at this temperature showed not only a very high degree of motility before freezing, but also a very substantially improved revival rate after freezing and thawing. Further work indicated that the best results were obtained if the semen was equilibrated in this way with a 10 % rather than a 15 % glycerol solution. Variations in the rate of freezing seemed to have little effect, although optimal revivals occurred if the rate of cooling between 5° C. and —15° C. did not exceed 2° per minute. Below —15° C. more rapid cooling was permissible.

These improvements in technique looked most encouraging, and experiments were therefore resumed on artificial insemination. The following procedure was adopted: semen immediately after collection was diluted with an equal volume of yolk-citrate buffer at about 30° C. and allowed to cool to 5° C. in the refrigerator over the space of 4 to 6 hours. It was then further diluted with an equal volume of citrate buffer containing 20 % glycerol, thus giving a final concentration of 10 % glycerol in the whole preparation. This was allowed to stand overnight (generally for 12 to 20 hours) at 5° C. On the following day,



1 ml. portions were transferred to small glass ampoules, maintained at 5° C. in a water-bath, which were sealed and cooled slowly over 45 mins. from 5° C. to -79° C. Samples were stored in solid CO<sub>2</sub> and alcohol for periods varying from 2 hours to 8 days. Ampoules were thawed as required immediately before use by immersing them in a water-bath at 40° C. 38 cows were inseminated with semen treated in this way: 6 of these returned on oestrus within 3 weeks, and of the remaining 32 which were examined for pregnancy 6 weeks following insemination 30 were found to be in calf, a pregnancy rate of 79 % (Polge and Rowson, 1952).

#### *Discussion.*

The experiments described are still in active progress, but even now, several general conclusions can be drawn from the work. Firstly, it has been shown that the addition of glycerol to bull semen does not decrease the fertilizing capacity of the spermatozoa. In fact, in the experiment involving insemination of unfrozen semen containing 15 % glycerol a pregnancy rate of 76 % was obtained, as compared with 50 % for semen containing no glycerol. This result strongly suggested, though not quite with statistical significance on the small number of cows involved, that the addition of glycerol improved the effectiveness of normal semen. Further work to get more information on this point is now being carried out. Secondly, it has been conclusively shown that the freezing of bull spermatozoa at -79° C. under certain conditions is compatible with the retention of fertilizing capacity. On the other hand, the frozen semen was diluted at the rate of only 3:1, and far more spermatozoa were used for each insemination than would normally be the case. It is not possible at this stage, therefore, to assess the number of spermatozoa which retain functional effectiveness after freezing and thawing. Work on semen frozen in higher dilutions is at present in progress, and the indications are that frozen and thawed semen will be quite effective in dilutions up to 1:40, which is the highest yet tried. The fact that a pregnancy rate with frozen semen was obtained which would be considered exceptionally good for normal semen in any concentration, considered in the light of the previous result, adds

weight to the indication that glycerol, in itself, may be exerting some beneficial effect on the spermatozoa.

As regards possible duration of storage at  $-79^{\circ}\text{C}$ ., only small numbers of cows have yet been inseminated with semen kept frozen for longer than 8 days. Nevertheless, preliminary results from experiments now in progress indicate that pregnancies can be obtained from semen stored at  $-79^{\circ}\text{C}$ . for 20 weeks, which is as far as the long-term experiments have gone at present. However, in view of the theoretical consideration that at so low a temperature time should be of little significance, this new technique can hardly fail to increase greatly the length of time for which bull semen can be preserved in a potentially functional condition. In support of this it has already been shown that fowl spermatozoa are capable of retaining their fertilizing capacity, at least to some extent, for as long as 9 months in the frozen state (*Polge and Parkes, 1952*). All semen samples which produced pregnancies in the insemination experiments described were thawed immediately before use. It might be convenient in certain circumstances to be able to use semen for several days after thawing. However, it is not yet known how long the frozen spermatozoa will retain their fertilizing capacity when thawed, although experiments are in progress to clarify this point.

This work has many significant implications both on the theoretical and practical side. The processes involved in the preservation of living cells during freezing and thawing are of great academic interest and some of the fundamental aspects are under investigation in the laboratories at Mill Hill. By microscopical observation it has been possible to follow the freezing and thawing of semen preparations both in the presence and absence of glycerol. Glycerol appears to modify the type of crystal formed in the medium during freezing so that less mechanical damage is caused to the cells (*Smith, Polge and Smiles, 1951*). On the other hand, our present knowledge concerning changes taking place within the cells is negligible. The work on bull semen raises questions on the permeability of cells both in relation to their reactions to low temperature and to changes in osmotic pressure and salt concentrations taking place during the process of freezing.

On the practical side, the possibility of storing semen for prolonged periods can hardly fail to have important applications in the artificial insemination of farm animals. If this technique becomes widely applicable it should prove useful in preventing much wastage of semen, which is unavoidable under present conditions, as well as having valuable applications in progeny-testing schemes and in permitting the fullest use of proven sires. Its use in permitting the transport of semen to any part of the world and also perhaps in the control of certain diseases transmitted through semen, are valuable potentialities.

In conclusion it should be emphasised that this paper is written at a time when much of the work is in its early stages. The cows described in these experiments which are pregnant to frozen semen have not yet produced their calves. However, normal calves have been born to cows inseminated with unfrozen semen treated with glycerol, and there is already one report of a calf having been produced from frozen semen (Stewart, 1951). A lot more information may be needed to determine optimal conditions for the prolonged storage of semen at low temperatures, but it is hoped that further results will be available by the time the Congress meets in July.

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#### *Summary.*

1. A technique is described for the preservation of bull semen frozen in solid carbon dioxide at  $-79^{\circ}\text{C}$ . The survival of the spermatozoa under these conditions is dependent upon the use of diluents containing glycerol.
2. Optimal conditions for freezing are: i) Dilution of the semen at  $5^{\circ}\text{C}$ . with a citrate buffer containing glycerol to give a concentration of 10 % glycerol in the semen. ii) Equilibration of the semen and diluent for 12 to 20 hours at  $5^{\circ}\text{C}$ . before freezing. iii) Cooling the semen slowly at



a rate which should not exceed 2° per minute between 5° C. and — 15° C., although below — 15° C. the rate may be quicker. Up to 80 % of the spermatozoa may resume motility after freezing by this technique and thawing at 40° C.

3. In 21 cows inseminated with unfrozen semen containing glycerol a pregnancy rate of 76 % was obtained.

4. 38 cows inseminated with glycerol-treated semen, which had been frozen at — 79° C., gave a pregnancy rate of 79 %.

5. In preliminary experiments on long-term storage, pregnancies have been obtained from semen kept frozen at — 79° C. for 20 weeks.

6. The possibility of very prolonged storage of semen and its application to artificial insemination is discussed.

The work described forms part of an investigation into the effects of very low temperatures on living cells being carried out in the Division of Experimental Biology at the National Institute for Medical Research, London. The authors wish to thank Dr. A. S. Parkes, F.R.S., and Dr. A. U. Smith for their help and encouragement. The experiments on the fertilizing capacity of frozen bull semen were undertaken at the Artificial Insemination Centre, Cambridge, and the authors are indebted to the Committee of the Cambridge and District Cattle Breeders Ltd., for their co-operation and to Mr. D. L. Milne who carried out many of the experimental inseminations. The expenses of this work were borne jointly by the Medical Research Council and the Agricultural Research Council.

#### Conservation à long terme de semence de taureau frigidifiée à des températures très basses (— 79° C).

##### Résumé.

1. Nous décrivons une technique par laquelle on peut préserver la semence du taureau par l'acide carbonique solidifié, à une température de — 79° C. La survivance des spermatozoïdes dans ces conditions dépend de l'emploi de délayants qui contiennent de la glycérine.

2. Les meilleures conditions de congélation sont les suivantes: (i) La dilution de la semence à une température de 5° C au moyen d'un tampon de citrate contenant assez de glycérine pour produire une concentration de 10 % de glycérine dans la semence. (ii) La stabilisation de la semence et du délayant pour une période de 12 jusqu'à 20 heures à une température de 5° C. avant de faire geler la semence. (iii) Le refroidissement graduel de la semence à une vitesse qui ne devrait pas dépasser 2 degrés par minute entre les températures de + 5° C et — 15° C; on peut cependant accélérer ce refroidissement aux températures au dessous de — 15° C. Jusqu'à 80 % des spermatozoïdes peuvent reprendre la motilité après avoir été gelés par cette technique et dégelés à une température de 40° C.

3. Après l'insémination de 21 vaches avec la semence non gelée et contenant de la glycérine, 76 % sont devenues pleines.

4. Après l'insémination de 38 vaches avec la semence contenant de la glycérine et gelée à une température de — 79° C, 79 % sont devenues pleines.

5. Dans nos expériences préliminaires au sujet de la conservation prolongée de la semence, nous avons trouvé que des vaches qui avaient été inséminées avec de la semence gardée pendant 20 semaines à une température de  $-79^{\circ}\text{C}$  sont devenues pleines.

6. Nous discutons la possibilité de conserver la semence pour une très longue période et d'appliquer cette technique à l'insémination artificielle.

**Langdauernde Aufbewahrung von gefrorenem Bullensperma  
bei sehr niedrigen Temperaturen ( $-79^{\circ}\text{C}$ .)**

*Zusammenfassung.*

1. Die Verf. beschreiben eine neue Technik zur Aufbewahrung von Bullensperma, welches in festem Kohlendioxyd bei einer Temperatur von  $-79^{\circ}\text{C}$  gefroren wird. Das Überleben der Spermien unter diesen Bedingungen hängt von dem Hinzufügen von Glycerin zu den Verdünnungsflüssigkeiten ab.

2. Das Gefrieren wird am besten unter folgenden Bedingungen ausgeführt: I) Verdünnung des Samens bei  $5^{\circ}\text{C}$  mit einer Glycerin enthaltenden Citrat-Pufferlösung um eine Konzentration von 10 % Glycerin in dem Samen zu erwirken. II) Equilibrierung des Samens mit der Verdünnungsflüssigkeit in 12 bis 20 Stunden bei einer Temperatur von  $5^{\circ}\text{C}$  vor dem Gefrieren des Samens. III) Langsames Abkühlen des Samens; zwischen  $+5^{\circ}\text{C}$  und  $-15^{\circ}\text{C}$ , nicht mehr als 2 Grad pro Minute, bei Temperaturen unter  $-15^{\circ}\text{C}$  kann das Abkühlen doch beschleunigt werden. Mittels dieser Technik können bis zu 80 % der Spermien wieder bewegungsfähig gemacht werden, nachdem sie bei einer Temperatur von  $40^{\circ}\text{C}$  wieder aufgetaut worden sind.

3. Von 21 Kühen, die mit Glycerin enthaltenden ungefrorenem Samen inseminiert wurden, wurden 76 % trächtig.

4. Von 38 Kühen, die mit Glycerin behandeltem und bei einer Temperatur von  $-79^{\circ}\text{C}$  gefrorenem Samen inseminiert wurden, wurden 79 % trächtig.

5. In vorläufigen Versuchen über langdauernde Aufbewahrung haben die Verf. gefunden, dass Insemination mit gefrorenem Sperma, welches in 20 Wochen aufbewahrt wurde, noch in Trächtigkeit resultierte.

6. Zum Schluss diskutieren die Verf. langes Aufbewahren des Spermas und die mögliche Anwendung bei der künstliche Besamung.