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Osmotic Pressure of Extended Bovine Semen During Storage

JOHN T. SMITH, DENNIS T. MAYER
H. A. HERMAN

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ABSTRACT

Semen obtained from dairy bulls of several breeds was extended with diluting media used in the practice of artificial insemination and with media especially designed to alter one or more of the physical and chemical properties.

These extended semen samples were stored at 4 to 7° C. and evaluated daily for motility and osmotic pressure. Motility was rated by a standard method. Osmotic pressure was determined with a modification of the Hill-Baldes thermoelectric osmometer and expressed in terms of freezing point depression. Results of this investigation show that osmotic relationships are important in preparing diluting media. They show, further, that the diluting media which maintained osmotic pressure within the limits of -0.44 to -0.61 °C. (freezing point depression) were superior in the maintenance of motility in extended bull semen during a 10-day storage period.

Glucose in the diluting solution aids in maintenance of osmotic pressure within narrow limits during storage. Addition of antibiotics to a diluent resulted in the appearance of a rise in osmotic pressure during the first 48 hours of the storage period, but maintenance of osmotic pressure was at a slightly lower level than that obtained with the same diluent without antibiotics. If the diluting medium without antibiotics maintained osmotic pressure within the optimal range, addition of antibiotics did not change the osmotic pressure to the extent that it was no longer within the optimal range.

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Osmotic Pressure of Extended Bovine Semen During Storage[1]

JOHN T. SMITH,[2] DENNIS T. MAYER[3] AND H. A. HERMAN

INTRODUCTION

The practice of artificial insemination is not a new one. Arabs were reported practicing artificial insemination in the breeding of horses centuries ago. However, it was not until 1930 that the artificial insemination of cattle came into use. By 1938 more than one million cattle had been bred artificially in Russia. In May of this same year, the first artificial breeding society or unit was organized in America. In 1938, the first calf resulting from artificial insemination was born in the dairy herd at the University of Missouri.

From this meager beginning 15 years ago the practice of artificial insemination has progressed to the point where artificial insemination societies have been formed that cover a section of a state and diluted semen is shipped daily to inseminators. This progress has, to a large extent, resulted from improved semen diluents; wherein factors such as storage and transport temperatures, pH, electrolytes, protective factors in egg yolk, and energy sources have been investigated extensively and the results applied to the preparation of diluting media.

In order to meet the demands for semen, especially from more desirable bulls, it is the practice to dilute the semen extensively, sometimes as high as 1:150.

It has been suggested by Salisbury, *et al.* (1948), that in these more dilute solutions, the osmotic pressure of the diluent might be an additional factor to be controlled. Swanson (1949), observed that bovine spermatozoa are more sensitive to hypertonic solutions of sodium citrate than to hypotonic solutions. He offered as an explanation the fact that, as semen ages, lactic acid increases, resulting in an increase in osmotic pressure to aggravate an already incompatible condition in a hypertonic solution. It is the purpose of this investigation to study the changes in osmotic pressure oc-

[1]Animal Husbandry, Agricultural Chemistry and Dairy Husbandry Departments, cooperating; University of Missouri, Columbia.

[2]This report includes most of the material in a dissertation presented by J. T. Smith for the M. S. degree.

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curring in semen extended with different diluents and maintained under controlled but varied conditions for a prolonged storage period.

REVIEW OF LITERATURE

Literature pertaining to osmotic pressure of semen, expressed in terms of freezing point depression, was reviewed by Anderson (1945), wherein he cited mean values from three different publications. Mean values cited by Anderson were -0.609°C. (range -0.53 to -0.65°C.) for determinations by Bernstein and Sergin, -0.62°C. (range -0.54 to -0.73°C.) for data published by Roemmele and -0.66°C. for determinations by Milovanov.

Salisbury, *et al.* (1948) investigated the freezing point depression of semen specimens chilled immediately following collection and reported the freezing point depression of semen which occurred with the greatest frequency to be -0.55°C., with a mean of 0.587°C. In addition, Salisbury, *et al.* (1948) found a positive and significant correlation between spermatozoan count and the freezing point depression, showing that, as the concentration count decreased, the magnitude of the depression of freezing point increased.

The effect of hypotonic, isotonic, and hypertonic solutions at different hydrogen ion concentrations upon the motility of spermatozoa was studied by Emmens (1948). Emmens found (using rabbit spermatozoa) that, at a pH of 5.8 to 6.6, the cells were more sensitive to hypotonic than to hypertonic solutions. At a pH of 7.0 to 8.7 little difference was observed between the sensitivity of the cells to either hypotonic or hypertonic solutions; however, at a pH of 8.6 to 9.6, the cells were more sensitive to hypertonic solutions.

While conducting an investigation designed to adjust the proportion of egg yolk and sodium citrate in the diluting medium, Swanson (1949) found 5 percent sodium citrate to have an immediate adverse effect upon spermatozoan motility. However, a 1 percent solution of sodium citrate was tolerated better, though it failed to maintain motility and resulted in a high proportion of spermatozoa with coiled tails which moved backwards or in circles.

Pursley and Herman (1950) studied the effect of hypotonic and hypertonic solutions of sodium citrate on the motility, livability and morphology of bovine spermatozoa. From data obtained as a result of this investigation, they established the optimum freezing point depression of diluters as -0.44 to -0.61°C.

The osmotic constants of semen and semen diluents cited above have been established through the use of the cryoscope. However, the standard Beckman cryoscope has the disadvantage of requiring a 20 ml. sample for each determination. Salisbury, *et al.* (1948) and Crawford and Nicosia (1952) have described cryoscopes which will measure the freezing point depression of smaller (5 ml.) samples; but, even a sample of only 5 ml. is undesirable when following the changes of osmotic pressure throughout a storage per-

iod. Because of the large volume required when the cryoscope was used for determining freezing point depressions, another method of measuring the osmotic pressure of semen which required less volume was sought.

Membrane osmometers requiring only a few drops of the fluids to be tested have been developed by Hansen (1952) and others. However, these were not tried, since, according to Aschaffenburg (1943), who investigated many membranes including collodion films impregnated with cupric ferrocyanide, there are as yet no truly semipermeable membranes. If, therefore, the membranes were not to be semipermeable to both electrolytes and colloids, the osmotic pressure obtained with them would not be the total osmotic pressure of the solution.

Baldes (1934) published a description of a micro-method of measuring osmotic pressure by the use of small thermocouples in the form of opposed loops. Baldes constructed the thermocouples of fine constantan and manganin wires, suspended them in a humidified chamber and connected them through copper leads to a high sensitivity galvanometer. He was able to show a linear relationship between the difference in the osmotic activity of the two drops on the opposed thermocouple loops and the deflection of the galvanometer. Later Baldes and Johnson (1939) published a detailed description of the method of construction of the thermoelectric osmometer, as well as a diagram of a jig on which to form the thermocouple. Fineman and McBain (1948) modified the thermocouples by replacing the manganin wire recommended by Baldes with one of fine copper. They had found that the manganin wire became brittle and broke under their service conditions.

The theory of the thermoelectric osmometer was derived by Baldes (1939) upon the assumption that the drops were spherical, and was experimentally justified by comparing the calculated (T-To) of 1 gram of NaCl per 100 grams H_2O (T) and distilled H_2O (To) with the experimentally observed value. According to Baldes a comparison of the calculated value 0.067°C. with the experimental value 0.065°C. indicated an efficiency of over 95 percent for the thermoelectric method.

Factors which would affect the accuracy of the thermoeletric osmometer were investigated by Roepke (1942). From his data Roepke was able to show that the accuracy of the method was not affected by size of the drop, surface active agents, viscous solutions, or small amounts of volatile solutes. It was his conclusion that the method is as accurate as the degree of accuracy with which the vapor pressure of the reference solution was known; the calculated probable error was 0.0003°C. in terms of freezing point depression.

Lifson and Lorber (1945) derived an equation which indicated the existance of the steady state in less than a minute and they experimentally verified this equation. They suggested that it was necessary for the readings to be made after a standard time interval and described a method of determining the osmotic pressure of a sample in five minutes by the thermoelectric method.

MATERIALS AND METHODS

Collection and Transport of Semen

The semen used in this investigation was collected by use of the artificial vagina, from healthy dairy bulls, maintained as a part of the Missouri Station dairy herd, and representing the three dairy breeds of Holstein, Jersey and Guernsey. Immediately after collection the semen was cooled to 20°C. at which temperature it was transferred to the laboratory.

Evaluation of the Semen

Upon arrival at the laboratory, the semen was evaluated for motility and freezing point depression. Motility was rated on a scale of 0 to 5 according to the method of Swanson and Herman (1941) under 100X magnification at a temperature of 98°F. A motility rating of 5 on this scale represents a highly motile specimen exhibiting waves and eddies strong enough to present a churning action under the microscope; whereas a motility rating of zero represents a specimen with no observable motility at 100X.

Freezing point depression was measured using a modification of the Hill-Baldes thermoelectric osmometer, modified and constructed in this laboratory. The modification of the osmometer incorporated the following criteria for satisfactory performance: (1) ease of sample transfer, (2) adaptability to a series of determinations, and (3) changing of the sample in a comparatively humid atmosphere. The design, shown in Figure 1, was considered to be a fulfillment of the above three criteria. This osmometer was constructed by firepolishing one end of a Pyrex glass tube, 15 mm. in diameter, to obtain an inward turned edge. This edge could be ground later as a seat for the plunger, which, when in its lowest position, would seal the humid measuring chamber air tight and stop convection currents. About 50 mm. from the top of the tube, which was 290 mm. in length, two side arms (5/16 O.D., Pyrex,40 mm. long) were sealed at an upward angle of 30°. A rubber stopper, selected to fit the 45 mm. I.D. jar which was used as a humid measuring chamber, was bored and the osmometer tube pushed through it until the seat end was flush with the bottom of the rubber stopper..

The plunger, which carries the thermocouple and its leads and seals the humid measuring chamber when in the lowered position, was made by blowing a pear shaped bulb on one end of a 5/16″ diameter glass tube just large enough to fit into the tube described above. Later the bulb was ground with 400 carborundum to fit against the lower or seal end of the tube. A portion of the bottom of the bulb was removed to allow passage of two large copper leads (No. 17 P. E. copper wire).

Construction of the thermocouple was greatly facilitated by the use of a jig, as described by Baldes and Johnson (1939) with the exception that the nails around which the thermocouple was formed were 2 mm. in diameter.

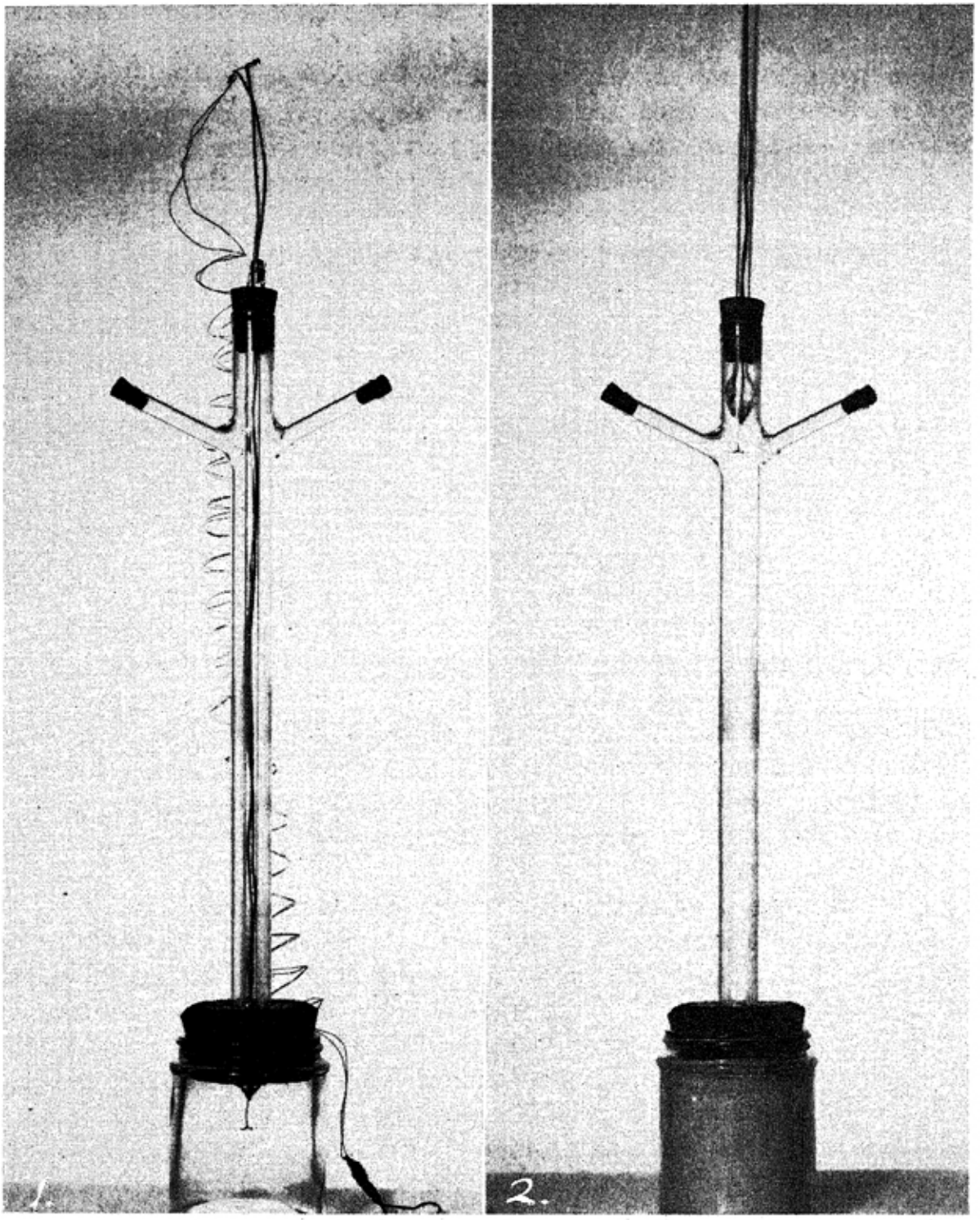


Figure 1—Osmometer with thermocouple in measuring position within the humid measuring chamber.

Figure 2—Osmometer is pictured with thermocouple raised to position for sample transfer.

All joints were soft soldered, using a rosin flux solder. The two wires used in the thermocouple were .001″ diameter constantan and .005″ diameter, copper, plain, enameled, magnet wire.The leads from the thermocouple copper wire were soldered to the leads in the plunger, after which the thermocouple was adjusted for position by raising or lowering the leads in the plunger. The plunger, thermocouple and leads were sealed together by the application of varnish, followed by baking in an oven at 120°C. This procedure was repeated until a good seal was achieved.

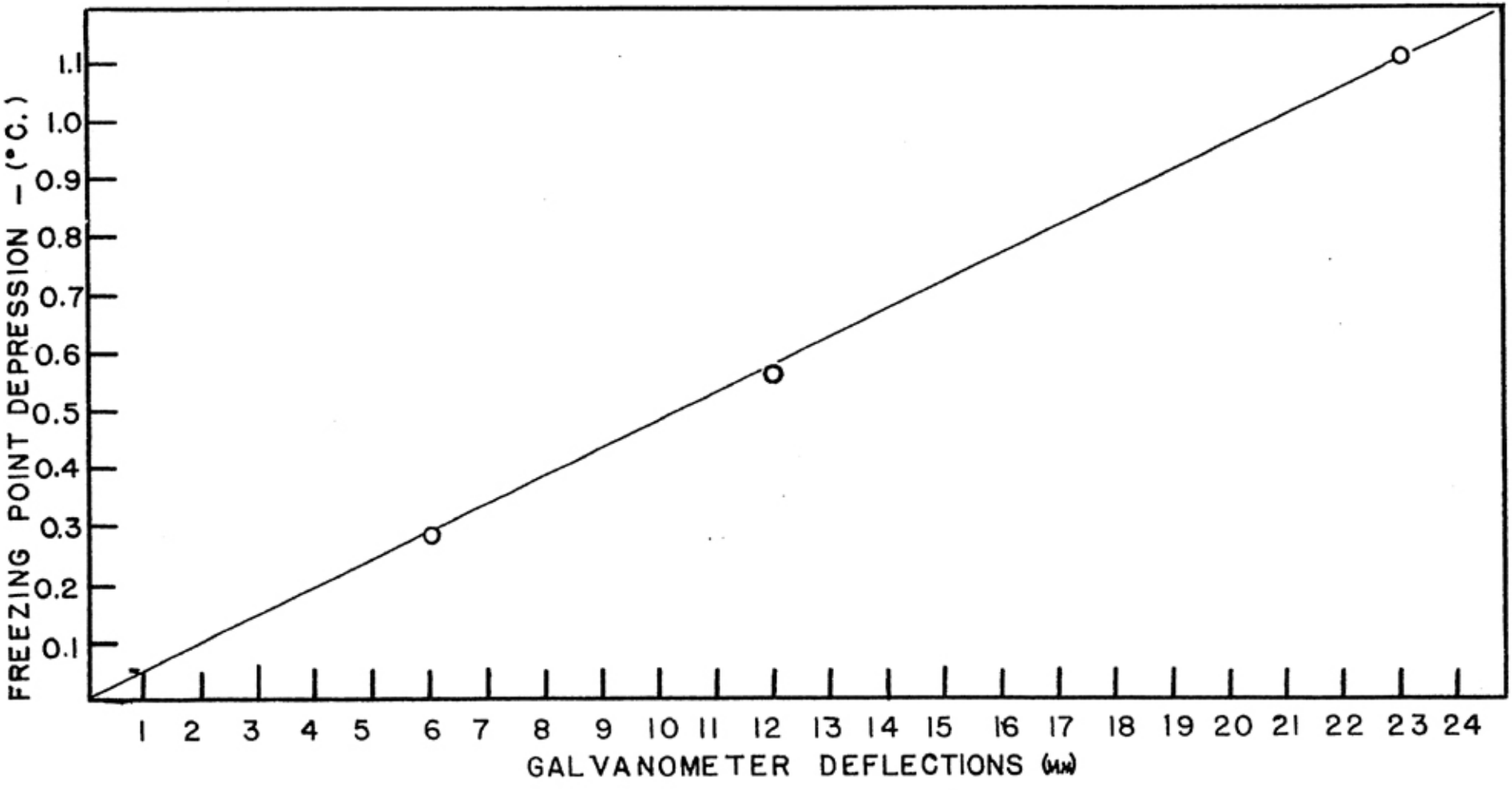
Leads to the galvanometer were wound from fine wire (No. 28 to 30) in the form of a spiral in order to permit raising and lowering of the plunger. These spiral leads were connected to the galvanometer by a double-pole, double-throw switch, the other side of which was connected to a circuit designed as a daily check on the galvanometer sensitivity, described by Baldes and Johnson (1939).

Deflection of the galvanometer was determined by use of a modified lamp and scale arrangement. The galvanometer must be a high sensitivity instrument because of the small voltages obtained, 2.5×10^{-6} volts when 0.9 percent NaCl is compared with distilled H_2O (Baldes, 1934). A Leeds and Northrup No. HS2284.B (sensitivity .05 uV 1 mm.) was the type of galvanometer chosen.

Thermostatic control of the apparatus was achieved by use of a modified Parkinson (1937) constant temperature gas regulated water bath, which by virtue of using a large volume of water and a sensitive thermostat to regulate the gas supply to a microburner placed beneath the water container, gave a water bath with constant temperature. The temperature control reported by Parkinson was ±0.0003°C.

Storage and Evaluation of Semen During Storage

The diluted semen samples were stored at 5°C. in small vials (14 x 50 mm.) which were placed in a large vessel containing 300 to 400 ml. of water. Not only did the large volume of water insure slow cooling of the diluted semen, but it prevented abrupt changes in temperature while the samples were being examined. Motility was rated and freezing point depression determined daily by use of the methods previously described. Figure 2 shows the osmometer with the unit (plunger-thermocouple-leads) raised to the position which it occupied when the samples were transferred. A sample transfer was accomplished by the following procedure. First, with the thermocouple raised to the position shown in Figure 2, the thermocouple loops were blotted clean with a strip of filter paper inserted through the side arms. The thermocouple loops were rinsed with a fine stream of distilled water from a small wash bottle and blotted as before. Then the sample solution and reference solution (0.15 M KCl) were placed on the blotted thermocouple loops by a curved-end capillary sampling pipette inserted through the side arms. The unit was lowered into position in the humid measuring



Graph 1—Calibration curve for thermocouple.

chamber as shown in Figure 1. The jar, which was the humid measuring chamber, was lined with filter paper as shown in Figure 2. The filter paper was moistened with water to keep the chamber humid during actual measurements.

Calibration of the Thermocouple

Calibration of the thermocouple was necessary in order that the galvanometer deflections could be evaluated in terms of freezing point depression. Calibration was achieved at the thermostated temperature of 25°C., using reference solutions of KC1. KC1 was chosen as the reference solution in this investigation because its osmotic coefficients have been extensively investigated and are reported in "the Physical Chemistry of Electrolytic Solutions" by Harned and Owen (1943). A plot of galvanometer deflections vs. calculated freezing point depression ($\triangle$ Tf) gave the expected linear relationship as shown in Graph 1. A linear relationship from a plot of galvanometer deflections vs. freezing point depressions was expected in view of the narrow range in temperature for which the thermocouple was calibrated.

RESULTS

Comparison of Values Obtained with Thermoelectric Osmometer Against Calculated Values and Values Obtained by Other Investigators Using Cryoscope

To check reliability of the new method, several substances were tested, for which the freezing point depression ($\triangle$ Tf) had either been determined by use of the cryoscope or could be calculated.

Results of this investigation are given in Table 1. Some of these results represent only a few determinations, but are included as an indication that

TABLE 1 -- COMPARISON OF OSMOTIC PRESSURE EXPRESSED AS $\Delta\pi$ (FREEZING POINT DEPRESSION) OBTAINED FROM HILL-BALDES OSMOMETER AND CRYOSCOPIC DATA OR CALCULATION

		Calculated		Cryoscope	
Compound	Hill-Baldes Osmometer	Complete Dissociation	Dissociation Constants	Value	Investigator
1.3% $NaHCO_3$	-0.370	-0.575	-0.290		
5% Glucose	-0.625	-0.515		-0.597	Moore & Mayer (1941)
17% Sucrose	-1.030	-0.920	-0.960		
Egg yolk	-0.670			-0.42	Howard (1944)
Egg yolk				-0.60*	Romanoff & Romanoff (1949)
Bovine semen	-0.545 (average of 28 ejaculates)			-0.587	Salisbury (1948)
Bovine semen				-0.609*	Anderson (1945)
Ram semen	-0.598			-0.590	Moore & Mayer (1941)
Ram semen				-0.641*	Anderson (1945)
Milk	-0.520			-0.550	A.O.A.C. (1950)
Milk				-0.530	Paley & Tzall (1951)

*Reporting averages of early workers.

the instrument has been calibrated at the proper values. It is perhaps disturbing to notice the high freezing point depression value for egg yolk, -0.67°C., when comparison is made with the value of -0.42°C. reported by

Howard (1944). However, it is in fair agreement with the value of -0.60°C. given by Romanoff and Romanoff (1949), especially when the viscosity of the material after exposure to air is considered. The rest of the values agree with values reported by at least one of the investigators cited.

Change in Osmotic Pressure Occurring During a 10-Day Storage Period with Different Diluents at a Dilution of 1:10

Since it was suggested in the Introduction that the changes which occurred during the prolonged, 10-day storage of semen were of primary importance, this phase of the problem is supported by the largest number of determinations.

Several diluents were used in this investigation. Among these was a lyophilized sample prepared on a large scale (A) and a standard diluter, previously developed in this laboratory by Kampschmidt, *et al.* (1951), which will be known as 524 in this investigation. The formulae of these and the other diluents used may be obtained by reference to Table 2. The diluents

TABLE 2 -- FORMULAE OF DILUENTS

Diluter Number	Chemical Components					
	Egg yolk	D.D.H_2O	5% Glucose	17% Sucrose	1.3% $NaHCO_3$	4.5% $Na_3C_6H_5O_7 \cdot 5\frac{1}{2}$ H_2O
635	10 ml.		20 ml.	20 ml.	10 ml.	
524	10 ml.		40 ml.		10 ml.	
500	10 ml.	14 ml.		26 ml.	10 ml.	
400	10 ml.	12.5 ml.	27.5 ml.		10 ml.	
200	20 ml.					20 ml.
A	Lyophilized product prepared commercially on a large scale.					

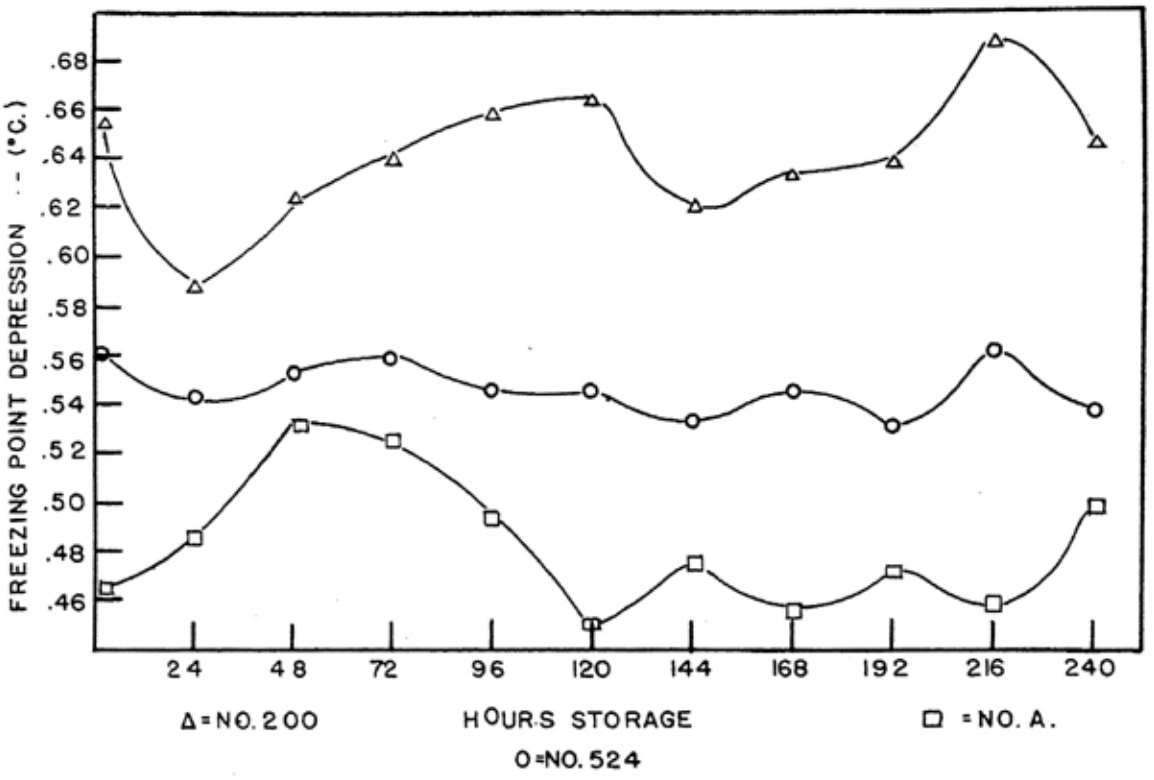
listed in Table 2 were used to extend semen in the ratio of 1 part semen to 9 parts diluter. Following dilution, the extended semen samples were stored and evaluated for motility and freezing point depression as previously described.

Data obtained from this part of the investigation are tabulated (Table 3) and illustrated in Graphs 2 and 3. Graph 2 illustrates the comparative

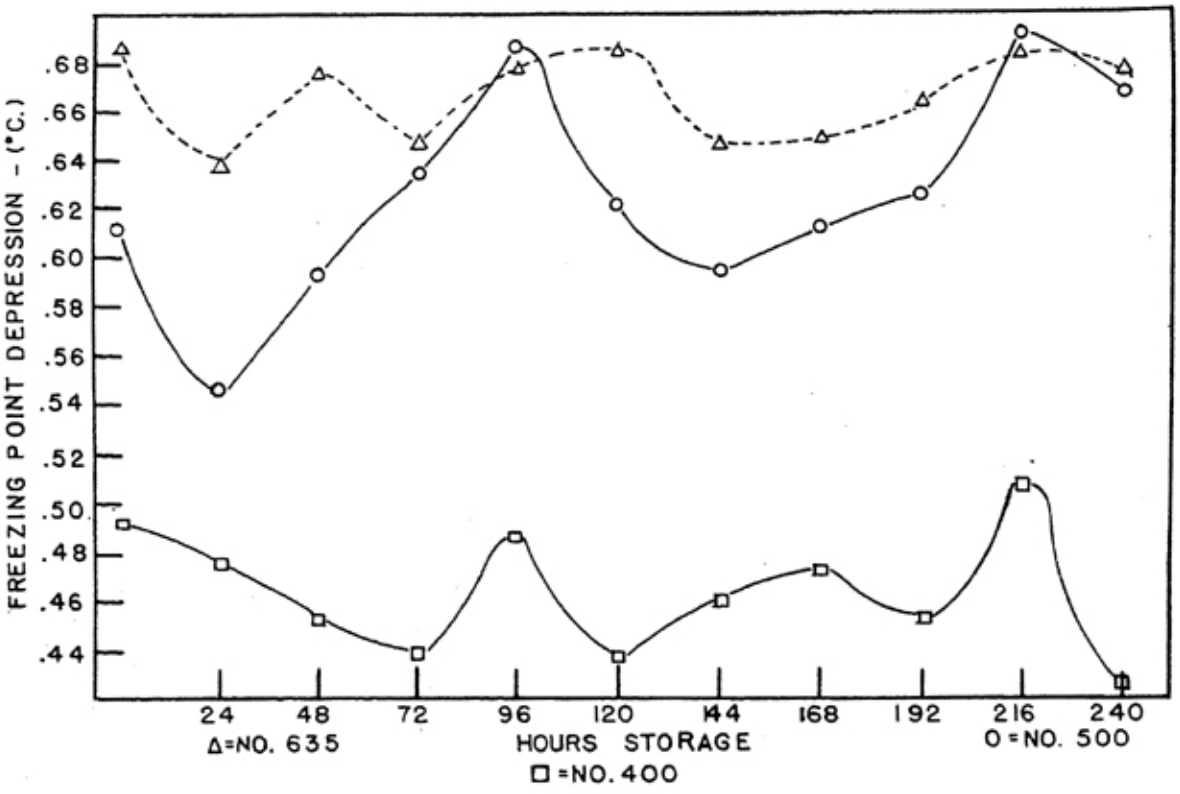
TABLE 3 -- MEAN VALUES OF ΔTf (FREEZING POINT DEPRESSION) AND MOTILITY DURING STORAGE OF SEMEN EXTENDED AT DILUTIONS OF 1:10

Diluter	Number Trials		Hours Storage 0	24	48	72	96	120	144	168	192	216	240
635	8	ΔTf	.685	.637	.675	.647	.678	.684	.645	.649	.664	.684	.676
		Motility	2.8	2.6	2.1	2.1	2.0	1.8	1.8	1.5	1.2	0.9	0.6
524	10	ΔTf	.561	.542	.551	.558	.547	.546	.534	.545	.529	.561	.536
		Motility	2.8	2.8	2.8	2.7	2.5	2.4	2.3	2.3	2.2	2.2	2.2
500	5	ΔTf	.611	.547	.594	.635	.687	.620	.594	.613	.625	.691	.667
		Motility	3.0	3.0	2.4	2.2	2.0	2.0	1.8	1.4	1.2	0.8	0.8
400	7	ΔTf	.492	.478	.455	.440	.488	.438	.462	.474	.455	.509	.421
		Motility	2.7	2.7	2.6	2.4	2.3	2.3	2.3	2.0	1.8	1.8	1.8
200	10	ΔTf	.658	.589	.626	.640	.659	.666	.620	.634	.638	.689	.646
		Motility	2.9	2.9	2.7	2.5	2.4	1.8	1.8	1.6	1.3	1.1	0.8
A	16	ΔTf	.466	.486	.532	.527	.494	.450	.476	.457	.472	.457	.498
		Motility	2.6	2.6	2.6	2.6	2.4	2.0	1.7	1.7	1.6	1.5	1.2

osmotic behavior of three diluters which have been used or recommended for use in the practice of artificial insemination. The nearly horizontal linear



Graph 2—Freezing point depression curves during storage of semen extended with diluents 200, 524, and A. Semen extended at a ratio of 1:10 (storage temperature 5°C).



Graph 3—Freezing point depression curves during storage of semen extended with diluents 635, 400 and 500. Semen extended at a ratio of 1:10 (storage temperature 5°C).

behavior of the Kampschmidt, *et al.* (1951) diluter along the line of mean freezing point depression of semen, is interesting in view of its superior motility maintenance. Graph 3 represents the comparative osmotic changes in semen extended with diluters containing different amounts of glucose and possessing different original △ Tf (freezing point depression) values. The erratic behavior of semen extended with diluter 500, a diluter which contained no glucose, in comparison with those specimens extended with glucose-containing media was striking.

Change in Osmotic Pressure Occurring During the 10-Day Storage Period at Dilutions of 1:50

Variation in osmotic pressure occurring during the storage of semen at higher dilutions was investigated, though not as extensively as the variation occurring at dilutions of 1:10. Only three of the diluents in Table 2 were used to extend semen in the ratio of 1 part semen to 49 parts of diluter, and only two determinations were run on each of the extended specimens. Results of this limited investigation have been tabulated (Table 4) and are

TABLE 4 -- MEAN VALUES OF △Tf (FREEZING POINT DEPRESSION) AND MOTILITY DURING STORAGE OF SEMEN EXTENDED AT DILUTIONS OF 1:50

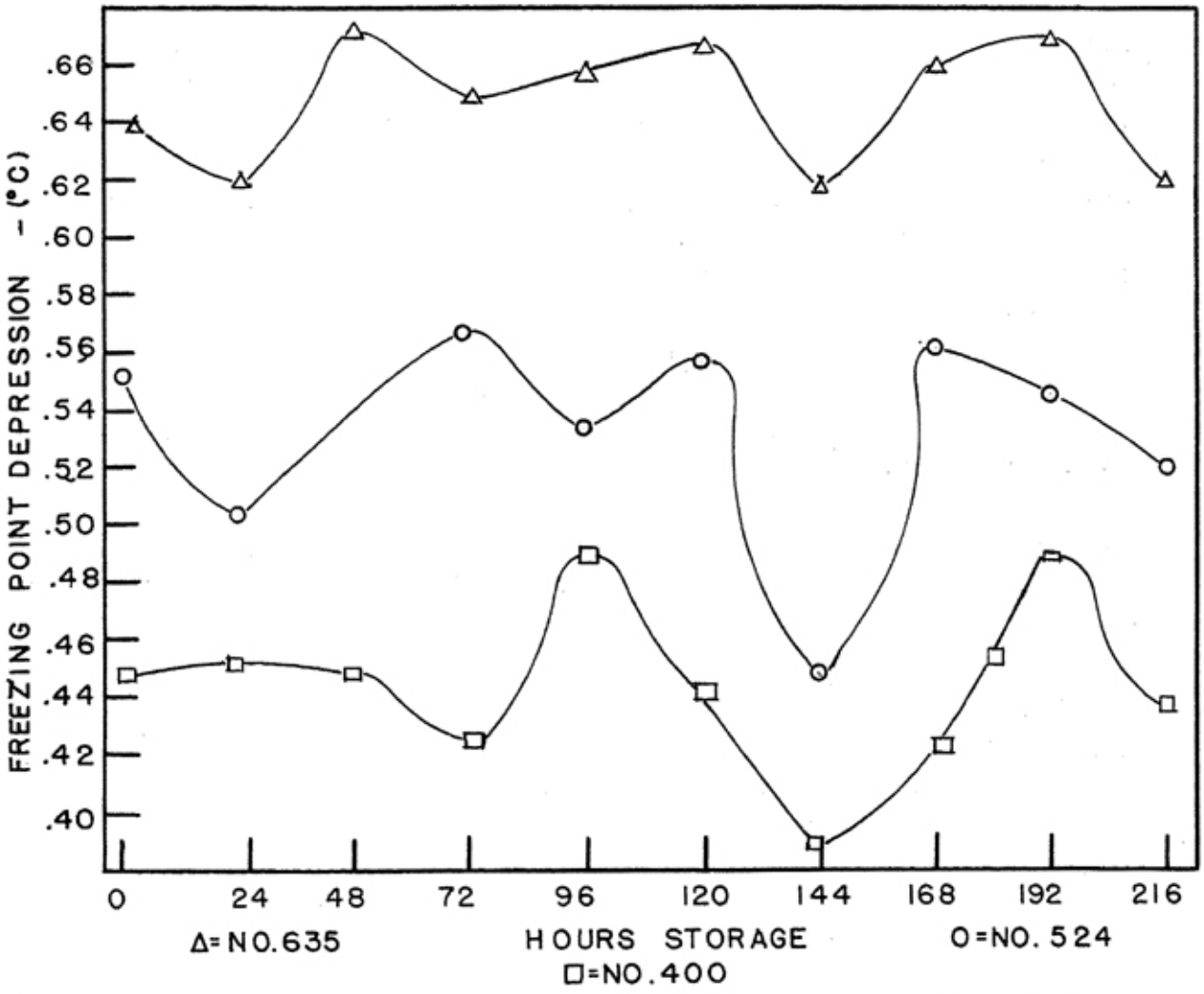
Diluter	Number Trials		Hours Storage 0	24	48	72	96	120	144	168	192	216	240
635	2	△Tf	.642	.619	.671	.649	.656	.666	.596	.659	.668	.619	
		Motility	2.5	2.5	1.2	1.2	1.2	1.2	1.2	0.5	0.5	0.5	
524	2	△Tf	.549	.503	.539	.566	.532	.556	.446	.561	.545	.519	
		Motility	2.5	2.5	2.0	2.0	2.0	2.0	2.0	2.0	1.2	1.0	
400	2	△Tf	.449	.452	.449	.426	.491	.440	.389	.424	.490	.436	
		Motility	2.5	2.5	2.0	2.0	1.2	1.2	1.2	1.2	1.2	1.2	

depicted graphically in Graph 4. These values do not vary greatly from those obtained at dilutions of 1:10. Since the evaluation of samples diluted in the ratio of 1:10 is much easier, this phase of the investigation was discontinued.

Effect of Incubation at 37.5°C. on the Osmotic Behavior of Extended Semen

Marr (1948), while studying the possibility of using the survival of semen after incubation at 37°C as a semen evaluation test, found a correlation between survival after 60 minutes incubation at this temperature and survival after 48 hours storage at 5°C. If, therefore, there is a correlation between the changes occurring during incubation and those during storage, an investigation of the osmotic pressure changes during incubation should give a pattern similar to that obtained during storage.

In this investigation five of the diluters listed in Table 2 were used to extend the semen, which was then incubated at 37.5° ±0.5°C. in a thermostated water bath. Thermostatic control of the bath was obtained by use of a mercury ether-bulb type thermostat which controlled the gas supply to a micro-burner placed beneath the bath. The small sample tubes (14 x 50 mm.) were held in centrifuge tubes in the bath which helped to maintain an



Graph 4—Freezing point depression curves during storage of semen extended with diluents 635, 400 and 524. Semen extended at a 1:50 ratio (storage temperature 5°C).

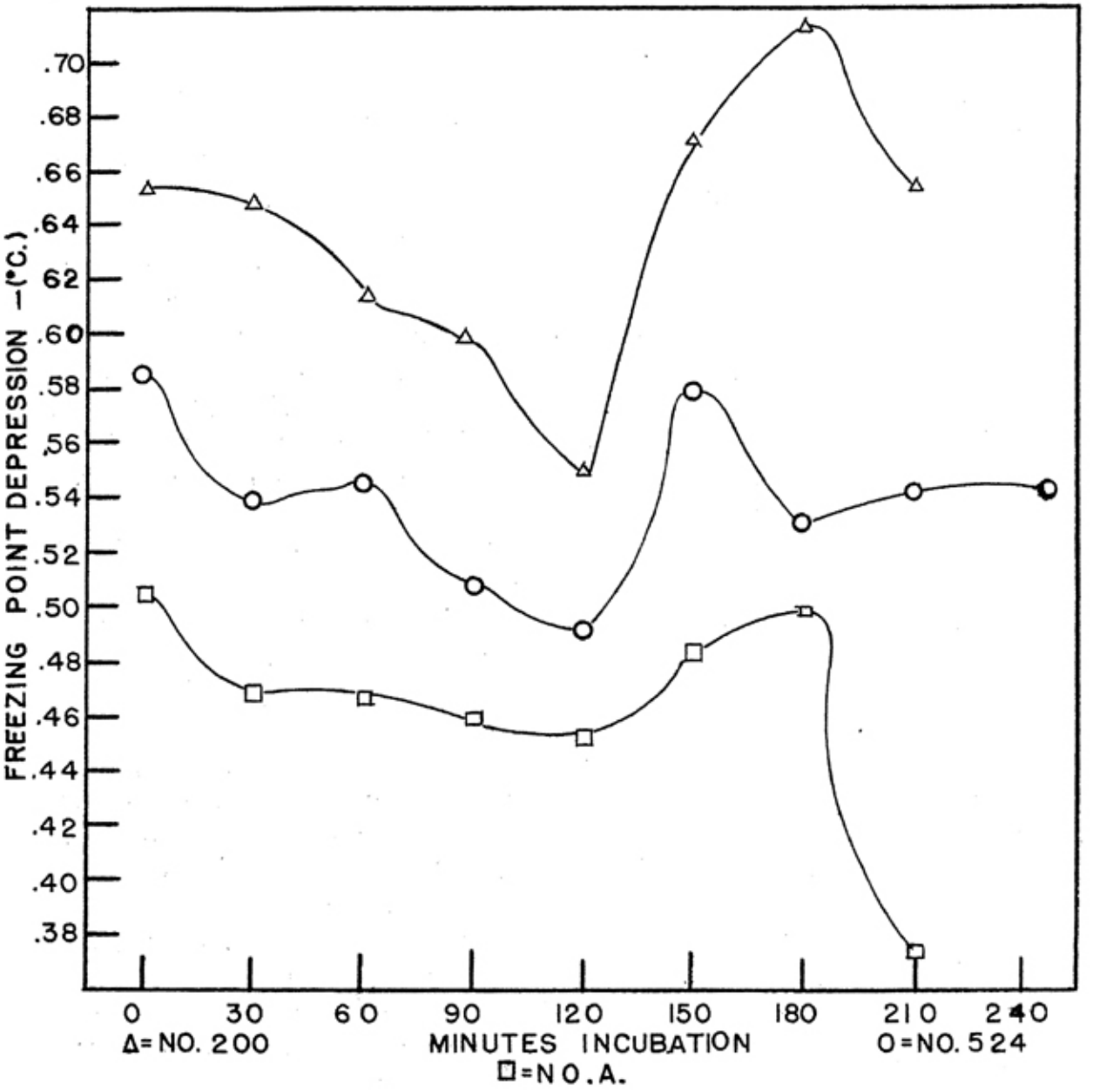
even temperature and hold the tubes upright. The samples were evaluated at regular intervals, usually 30 minutes, for both motility and △ Tf (freezing point depression).

TABLE 5 -- MEAN VALUES OF ΔTf (FREEZING POINT DEPRESSION) AND MOTILITY DURING INCUBATION OF EXTENDED SEMEN AT 37.5°C. ± 0.5°
DILUTION RATE 1:10

Diluter	Number Trials		Minutes Incubation 0	30	60	90	120	150	180	210	240
635	1	Δ Tf	.662	.695		.660		.718			.731
		Motility	2.+	2.+		2.+		2.+			2.0
524	3	Δ Tf	.587	.539	.545	.507	.491	.580	.531	.541	.543
		Motility	3.0	2.6	2.6	2.0	2.0	2.0	2.0	2.0	2.0
400	1	Δ Tf	.531	.463		.454		.454			.579
		Motility	2.0	2.0		2.0		2.0			2.0
200	3	Δ Tf	.654	.649	.613	.596	.550	.671	.713	.653	.661
		Motility	2.7	2.3	2.3	2.0	1.7	1.7	1.3	1.3	1.3
A	8	Δ Tf	5.06	.469	.469	.460	.454	.486	.499	.375	
		Motility	3.0	3.0	3.0	2.8	2.7	2.6	2.5	2.2	

Data obtained from this investigation are shown in Table 5, and the behavior of three of the more thoroughly investigated samples may be followed in Graph 5.

These data support the findings of Marr (1948), since they do not differ markedly from those obtained with samples under normal storage conditions.



Graph 5—Freezing point depression curves during incubation of semen extended with diluents 200, A, and 524. Dilution ration 1:10 (incubation temperature 37.5°C).

DISCUSSION AND SUMMARY

Since the major portion of the investigation consisted of a study of the osmotic behavior of six diluent mixtures, the formulae of which may be recalled by reference to Table 2, this discussion will be centered around the data obtained using these six diluters. Three of these diluters, numbers 524, 200 and A, have been or are being used in the artificial insemination of dairy cattle.

It has been a general practice, based upon the reports of numerous investigators, to prepare semen diluters which possessed an initial osmotic pressure similar to that of mammalian semen. Pursley and Herman (1950) stated that diluters would yield satisfactory results if their osmotic pressure was within a narrow range ($\triangle$ Tf of -0.44° to -0.61°C.).

Results of this investigation present an additional requirement of diluters for satisfactory semen storage. The results show that diluters which maintain the osmotic pressure of extended semen within the narrow range suggested by Pursley and Herman as the optimal initial osmotic pressure for diluters are those which also maintain viability, as measured by motility rating at a maximum throughout a 240-hour storage period. Diluters 524 and A maintained osmotic pressure with little change during the 10-day storage period and these two diluters were superior to the others investigated in maintenance of motility.

The difference between diluters 524 and A, with the exception that A contains added antibiotics, is that the egg yolk in A was lyophilized prior to the addition of the other dry ingredients. This diluter must be reconstituted by the addition of water before being used to extend semen.

In the consideration of plausible causes for the differences in osmotic pressure changes during the storage of semen extended with these two diluters, the fact that diluter A contained antibiotics suggests that the superior conrol of the bacterial population in semen extended with it may have influenced osmotic pressure levels. That the differences observed between the freezing point depression curves of diluent 524 and A might be due to the added antibiotics was strengthened by results obtained from the addition of 300 mg. percent of sulfanilamide to diluent 524. Addition of 300 mg. percent sulfanilamide to diluent 524 resulted in the appearance of a rise in the freezing point depression curve during the first 48 hours of storage, followed by a decrease with maintenance of osmotic pressure at a lower level than that obtained with diluent 524 without added antibiotics.

However, the possibility that the differences also might be due to factors resulting from the lyophilization of the egg yolk in diluter A was given consideration, because it was observed in this laboratory that lyophilized lecithin as a substitute for egg yolk gave a 48-hour peak in the freezing point depression curve of semen extended with it, similar to that obtained with semen extended with diluter A.

Diluter 200, the so-called egg yolk-citrate diluter, used quite extensively in the commercial practice of artificial insemination, is different from either diluter 524 or A, both in the buffer salt present and in the lack of added glucose. These diluters also differ in egg yolk:buffer ratio; the egg yolk:buffer ratio is 1:1 for the egg yolk-citrate diluter (200) and 1:5 for the egg yolk: buffer salt + glucose solution of diluter 524. Therefore, any attempted explanation of the difference in osmotic behavior should be made only after due consideration of these differences in diluter constitution.

The diluter containing sodium bicarbonate as a buffer has been shown to be superior to that containing sodium citrate for maintaining spermatozoan motility by Kampschmidt, *et al.* (1951), a point which is strenghthened by the observation that diluter 200 maintained an osmotic pressure above maximum limits of the range established by Pursley and Herman (1950) for almost the entire storage period. Only the 24-hour value (Table 3) was below the upper limit of the optimum range established by these workers. Diluter 200 also proved inferior to diluters 524 and A in motility maintenance.

Data on storage results at dilutions of 1:10 with three other diluters are included in Table 3. These were experimental diluters prepared by altering the constitution of the basic diluter 524 in an endeavor to gain further information regarding the superiority of this basic diluter. Alterations incorporated into these three experimental diluters were: (1) amount of added glucose; (2) addition of sucrose, instead of glucose, to meet osmotic requirements, diluter 500; and (3) an alteration of their original osmotic pressure or freezing point depression, diluters 635 and 400. It may be observed by reference to Table 2 that diluter 635 contains 20 ml. of 5 percent glucose, or ½ as much glucose as diluter 524, and that diluter 400 contains 27.5 ml. of 5 percent glucose and has a freezing point depression lower than diluter 635 by -0.2°C. Since its original osmotic pressure was purposely set high, diluter 635 maintains osmotic pressure above the limits set by Pursley and Herman (1950). However, the osmotic pressure was maintained within the narrow range of -0.64 to -0.68. The capacity of diluter 635 for maintaining osmotic pressure within a narrow range was similar to that of diluter 524. The results show that diluter 635 was inferior to 524 in the maintenance of motility in semen extended with it. These results indicate that osmotic pressure at hypertonic levels, even though the fluctuations in pressure are at a minimum, is harmful to bull spermatozoa under storage conditions.

Diluter 400, like diluter 635, contained added glucose but was purposely adjusted to a lower initial osmotic pressure. It maintained osmotic pressure within the limits established for satisfactory diluter performance (Pursley and Herman, 1950) with a range in freezing point depression of -0.44 to -0.51°C (Table 3). It showed only slightly greater fluctuations than diluter 524 with a range in $\triangle$ Tf of -0.53 to -0.56°C. Diluters 400, 524, and A

maintained osmotic pressure in extended semen within the range of freezing point depression of -0.44 to -0.51°C. Since these three diluters were superior to all others investigated in their capacity for maintaining spermatozoan motility during storage, it appears that the osmotic pressure of extended semen should be kept within this range.

Of the six diluters studied, four (635, 524, 400 and A) contained glucose as one of their major constituents. The remaining two (diluters 500 and 200) contained no glucose. All of the diluters contained sodium bicarbonate as the buffer salt, except diluter 200 which contained sodium citrate.

A comparison of the range of freezing point depression in semen extended with diluter 500 (-0.55 to -0.69°C.) with that of semen extended with diluter 200 (-0.59 to -0.69°C.), shows a rather wide fluctuation of osmotic pressure under storage conditions. On the other hand, the glucose containing diluters (635, 524, 400 and A) maintained osmotic pressure of extended bull semen within narrow limits even though these diluters maintained the osmotic pressure at different levels. Apparently, then, glucose plays some role in preventing marked fluctuations in osmotic pressure during the storage of extended semen.

Osmotic changes in bull semen extended with several of the six diluters listed in Table 2 and layered with mineral oil prior to storage were determined but the data has not been presented in this publication. Diluter 524, which maintained the osmotic pressure of extended semen within a narrow range under normal storage conditions, failed to do so under oil; the range being -0.50 to -0.63°C. Interestingly, semen extended with either diluter 500 or 200, neither of which contain glucose, yielded the same results whether layered with oil or not during the storage period. The significance of this observation is in doubt at the present time.

Interpretation of data obtained in the incubation experiments at 37°C. is difficult. In Table 5 and Graph 5 a decrease in osmotic pressure during the first 120 minutes of incubation, followed by a sharp increase, is apparent. Perhaps, the decrease may be caused by physical factors such as lowered Co_2 and O_2 tension at the higher temperature. It may be that the sharp rise after 120 minutes is due to increased bacterial activity. However, no evidence is available to substantiate either of these suggestions.

CONCLUSIONS

1. Osmotic pressure should be an important consideration along with other physical factors such as pH, buffering capacity, and viscosity in preparation of diluting media for bull semen.

2. It is not sufficient to use isotonic solutions or solutions having an osmotic pressure within a narrow range near that of semen as diluting media. Results of this investigation show that diluting media should maintain the osmotic pressure within the limits of -0.44 to -0.61°C. (expressed as freez-

ing point depression) for maximal motility maintenance during a 10-day storage period.

3. The presence of glucose in diluting media aids in the maintenance of osmotic pressure within narrow limits and eliminates the marked fluctuations observed in semen specimens diluted with media containing no glucose.

4. Osmotic pressure changes in semen extended at the ratio of 1:49 are similar to those observed in semen extended at the ratio of 1:10, hence dilution rate within this range has little influence upon osmotic changes during storage of extended bull semen.

5. Although antibiotics in the diluting medium may cause a rise in osmotic pressure early in the storage period, in the few cases observed in this investigation they had little effect upon the general trend of osmotic changes during a storage period. If a diluting medium maintained osmotic pressure within the optimal limits, addition of antibiotics did not alter this tendency.

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