LH as is seen following ovariectomy; not as much LH was in plasma at the same time on the afternoon of estrus. The 5-day cyclic rats had significantly more LH in plasma at both proestrus and estrus than was found in 4-day cyclic rats. Pentobarbital administered at 2 PM (at a dose level which blocks ovulation in the proestrous 4-day rat) reduced plasma OAA depleting activity to the minimal amount found in hypophysectomized rats.

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Factors Influencing Uptake of Triiodothyronine-I^{131} by Bull Spermatozoa.* (30087)

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Radioactive labeled glucose(1,2), fructose (3), phospholipids(4), glycerol(5), and amino acids(6) have been previously used to study the metabolic activity of bull spermatozoa. An earlier report(7) from this station indicated that there was an active *in vitro* uptake of radioactive triiodothyronine-I^{131} (T_3-I^{131}) by bull spermatozoa. It was found that uptake of T_3-I^{131} was related to viability of the sperm cells and the presence of seminal plasma and egg yolk in the suspending medium and that it was independent of the amount of triiodothyronine added to the diluted semen. The present report is a further investigation of the factors affecting uptake of T_3-I^{131} by bull spermatozoa.

Materials and methods. Semen from dairy bulls was used in 4 different trials. After diluting the semen to the desired sperm cell concentration the spermatozoa were washed 3 times by centrifugation (1140 *g*). The solutions used for washing and suspending the spermatozoa were 2.9% sodium citrate and a more complex ionic medium, the Norman and Johnson (N-J) solution(8) containing 500 mg% of glucose. Stock solutions of commercial preparations of radioactive triiodothyronine (Squibb) were diluted to 50 ml with psysiological saline solution and were used

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routinely for a period of 3 weeks. To determine the percent uptake of T_3-I^{131} by spermatozoa, 0.1 ml of the diluted isotope solution was added to each tube containing 1 ml of diluted and washed spermatozoa. This amount of isotope had an activity of 0.02 μc and a range of weight from 0.706×10^{-3} to 1.180×10^{-3} μg. Following the addition of isotope, the tubes were incubated in a water bath at 37°C with continuous gentle agitation. The radioactivity of the samples was determined after incubation using a well-type scintillation counter. The spermatozoa were then centrifuged (1579 *g*) and washed 3 times with 10 ml of sodium citrate solution or N-J solution. Following the washings the radioactivity of the spermatozoa was then recounted.

$$\% \text{ uptake} = \frac{\text{Net counts/min of spermatozoa after washing}}{\text{Net counts/min of spermatozoa before washing}} \times 100$$

Experimental procedures and results. Trial 1. *Effect of diluent, spermatozoa concentration and incubation time upon uptake of T_3-I^{131}.* Aliquot samples of semen from 2 bulls were washed and diluted in sodium citrate solution or N-J solution. These samples were then further divided and diluted in a factorial design to give 4 levels of cell concentration and 4 intervals of incubation time in the presence of T_3-I^{131}. The cell concentrations were 10, 80, 150, and 220 million per ml and incubation intervals were 15, 45, 75, and 105 minutes.

Spermatozoa uptake of T_3-I^{131} increased significantly ($P<0.01$) with increased cell concentration and incubation interval (Table I). A definite effect appeared with spermatozoa suspended in sodium citrate solution exhibiting a greater uptake of labeled T_3 ($P<0.01$) than those in N-J solution (21.1 *vs* 9.2%). A significant interaction ($P<0.05$), however, was found between diluents and sperm cell concentrations, which is illustrated in Table I by a relatively greater increase in T_3-I^{131} uptake by the cells suspended in sodium citrate solution than those suspended in N-J solution at each cell concentration.

A second experiment was designed to study further the effects of incubation time on uptake of T_3-I^{131} by spermatozoa diluted in the 2 solutions. The intervals studied were 15, 30, 60, 120 and 240 minutes and concentration of sperm cells was 100×10^6 per ml. The percentages of labeled T_3 taken up after the respective incubation intervals were 11.4, 15.6, 19.2, 23.2, and 30.8 for spermatozoa in sodium citrate solution and 7.8, 9.7, 12.6, 14.6, and 16.9 for spermatozoa in N-J solution. The results again indicate a difference in T_3-I^{131} uptake between spermatozoa suspended in the 2 diluents. There was also a definite trend for cellular uptake of T_3-I^{131} to level off after one hour in both diluents. Spermatozoa motility had dropped from 55% at 15 minutes for both diluents to 1% and 40% at 60 minutes for the sodium citrate and N-J solutions, respectively.

TABLE I. Effect of Diluent, Incubation Time and Sperm Cell Concentration on T_3-I^{131} Uptake by Spermatozoa.

Incubation time (min)	Diluent	Cell concentration (10^6) 10	80	150	220	Incubation time means
		Mean uptake, %				
15	Citrate	1.2	7.8	13.3	18.7	10.2
	N-J	1.3	3.9	6.2	8.7	5.0
45	Citrate	5.6	16.5	26.8	31.6	20.1
	N-J	3.2	9.0	7.7	13.3	8.3
75	Citrate	2.4	20.8	34.9	38.6	24.2
	N-J	2.3	5.5	13.2	16.5	9.4
105	Citrate	5.7	27.3	39.4	46.3	29.7
	N-J	2.9	10.8	18.7	24.3	14.2
Concentration means	Citrate	3.7	18.1	28.6	33.8	
	N-J	2.4	7.3	11.4	15.7	

Trial 2. *Effect of glucose concentration upon T_3-I^{131} uptake by bull spermatozoa.* One possible explanation for the diluent effect noted in Trial 1 was the presence in the N-J solution of glucose, an important metabolic substrate for spermatozoa. To determine the effect of glucose on T_3-I^{131} uptake by sperm cells each of 4 semen samples was split with one-half of the sample washed, diluted, and suspended in sodium citrate solution and the other half in N-J solution free of glucose. These samples were then further divided and diluted into 6 subsamples containing various levels of glucose and 100×10^6 sperm cells per ml. The subsamples were incubated with the labeled T_3 for one hour.

The percentages of T_3-I^{131} uptake by the spermatozoa in samples containing 0, 1, 2, 3, 4, and 5 mg of glucose per ml were respectively, 21.5, 21.1, 20.9, 21.9, 19.1, and 22.0 for cells in sodium citrate extender and 16.2, 15.9, 16.2, 14.6, 14.5, and 15.7 for spermatozoa in N-J solution. Again the spermatozoa diluted in sodium citrate solution had a significantly ($P<0.05$) greater uptake of T_3-I^{131} than those diluted in N-J solution. There was, however, no significant difference in T_3-I^{131} uptake among the glucose levels studied nor were there any interactions of special significance.

Trial 3. *Sperm cell death and T_3-I^{131} uptake.* Previous work(7) had shown that sperm cells killed by cold shock exhibited a lower uptake of T_3-I^{131} than untreated spermatozoa. Although death of the cell was attributed as the cause of the diminished uptake, another important possibility may have been the damage or destruction of the cellular membrane by the treatment. In this trial, sperm cell death, as determined by immotility was accomplished by several means. Four semen samples were washed twice in N-J solution, and the spermatozoa were suspended to 100×10^6 cells per ml. From each of the 4 samples, 6 1-ml subsamples were removed and centrifuged once again. The sperm cells in 5 subsamples were resuspended in 1 ml of N-J solution while the sixth subsample was resuspended in a glucose-free N-J solution. The latter subsample was then kept in a water bath at 38°C until the spermatozoa exhibited no motility (10 hr). The 5 remaining subsamples were kept at room temperature and at the time when motility of the incubated cells had ceased (death), these subsamples received the following treatments: none (control), cold shock by alternate rapid cooling and warming, 0.1 ml of 10% sodium hydroxide, 0.1 ml of 10% acetic acid, and 0.1 ml of 5% formaldehyde. The subsamples were incubated for one hour following their respective treatments and the addition of the labeled T_3.

The percentage of motile spermatozoa averaged 56 for the 4 control subsamples, while none of the treated cells showed any motility. Uptake values of T_3-I^{131} for the respective treatments were: control, 23.6%; cold shock, 7.4%; incubation, 13.2%; sodium hydroxide, 15.6%; acetic acid, 14.4%; and formaldehyde, 30.7%. Compared to the control samples, all of the treatments except formaldehyde caused a significant drop ($P<0.01$) in T_3-I^{131} uptake; formaldehyde significantly increased ($P<0.01$) uptake of T_3-I^{131}. The spermatozoa killed by cold shock had the lowest uptake of isotope, which was significantly less ($P<0.01$) than that of cells killed by continuous incubation, sodium hydroxide, or acetic acid. The latter 3 treatments having similar effects on uptake of T_3-I^{131} by sperm cells.

Trial 4. *Effect of diluent and storage interval upon motility and uptake of T_3-I^{131} by spermatozoa.* Each of the semen samples from 3 bulls was divided into 4 portions and diluted to 100×10^6 cells per ml. Two of the subsamples were diluted in N-J solution, one being stored at room temperature (22°C) and the other at 7°C. One of the remaining 2 subsamples was diluted in N-J solution plus 1% egg yolk and the other was diluted in sodium citrate solution plus 20% egg yolk. Both of these subsamples were stored at 7°C. All of the diluents contained 1000 units of penicillin and 500 μg of dihydrostreptomycin per ml. The samples were stored for a total of 18 days with motility estimations and T_3-I^{131} uptake assays made at 2-day intervals starting immediately after the samples had been diluted and properly cooled to their respective temperatures. Prior to incubation with the isotope for one hour the cells were washed and resuspended in N-J solution.

An analysis of variance of the data for both the percent of T_3-I^{131} uptake and motile spermatozoa indicated that semen quality was best maintained ($P<0.01$) in the egg yolk-sodium citrate medium (Table II). The addition of egg yolk to the N-J solution resulted in prolonged cell life and greater uptake of labeled T_3 ($P<0.01$). In all cases the greatest drop in percent of T_3-I^{131} taken up by the sperm cells occurred between day 0 and 2. This drop was 19, 43, 31 and 20% for cells suspended in N-J solution at 22°C, N-J solution at 7°C, N-J solution plus 1%

TABLE II. Effect of Storing Spermatozoa for 18 Days Upon Motility and Uptake of T_3-I^{131}.

Day of storage	Diluent and storage temperature: N-J, 22°C		N-J, 7°C		N-J + 1% egg yolk 7°C		Citrate + 20% egg yolk, 7°C	
	Motility	Uptake	Motility	Uptake	Motility	Uptake	Motility	Uptake
	Mean %		Mean %		Mean %		Mean %	
0	69	24.6	65	17.5	69	27.9	75	28.2
2	50	19.1	27	9.9	62	19.4	67	22.9
4	42	14.4	11	9.5	53	20.2	60	21.6
6	33	16.3	3	10.9	47	20.3	53	23.0
8	1	9.7	1	9.4	35	19.0	37	22.8
10	0	12.1	0	12.0	31	17.1	32	23.5
12	0	12.6	0	11.4	19	16.7	21	21.2
14	0	11.9	0	10.4	11	15.0	16	19.4
16	0	11.0	0	8.6	3	15.1	15	20.1
18	0	11.0	0	8.1	0	15.0	8	19.3

egg yolk at 7°C, and sodium citrate solution plus 20% egg yolk at 7°C, respectively.

Correlations among subclass means between the percentages of spermatozoa motility and T_3-I^{131} uptake were relatively high among the diluents, with an overall coefficient of 0.83 ($P<0.01$). The overall within-bull correlation coefficient between these 2 variables was 0.78 ($P<0.01$). These values indicate a fairly high degree of association of the T_3-I^{131} uptake of spermatozoa with their motility. A similarly calculated correlation between percent isotope uptake and day of storage had a coefficient of −0.79 ($P<0.01$).

Discussion. As was previously reported(7) and further substantiated by these trials, the uptake of T_3-I^{131} by bull spermatozoa is associated with the viability and motility of these cells. A diluent effect on the uptake of labeled T_3 by spermatozoa was evident throughout the trials. The 2 diluents used were selected on the basis that sodium citrate is a simple salt solution offering buffering capacity and is widely used with egg yolk for semen dilution and that the N-J solution is a more complex ionic medium containing glucose and capable of maintaining cell viability for an extended period. The addition of glucose to either diluent after the cells had been washed did not affect the uptake of T_3-I^{131} nor greatly influence cell survival in the sodium citrate solution. Sullivan and Mixner(7) reported that washing the spermatozoa with sodium citrate solution to remove the seminal plasma and the diluent containing egg yolk resulted in a markedly increased uptake of T_3-I^{131} by the cells. This was attributed to removal of certain complex organic constituents of these materials which competed with sperm cells in the binding of the T_3-I^{131}. N-J solution, however, does not contain these constituents and since it does cause a decreased uptake of T_3-I^{131} compared to sodium citrate solution, this suggests that the effect of seminal plasma and egg yolk was not due entirely to competitive binding of T_3 by their components but was also due to certain ions found in these diluents and N-J solution. The inhibitory effect of these ions on the labeled T_3 uptake could be reflected through changes in the metabolic activity of the spermatozoa(9) or in the integrity of the cellular membrane.

The difference in T_3-I^{131} uptake between live spermatozoa and those killed by continuous incubation, acetic acid, and sodium hydroxide probably indicates the relative amounts due to an active uptake by live cells compared to a passive uptake by dead cells. Another possibility is the loss of T_3 binding substances through leakage from the cell during the incubation, which could have contributed to the lower uptake by the dead cells. The lower uptake by the cold-shocked cells reflects the loss of the cellular binding substances through disruption of the cells. The large increase in T_3-I^{131} uptake by formaldehyde-killed spermatozoa may have been due to greater retention of binding substances in or on the cell and an increased binding strength of these constituents resulting from

the ability of formaldehyde to fix cells without appreciably changing their nature.

The final trial again indicated the association of T_3-I^{131} uptake with percentage of motile spermatozoa in a given sample. All treatments exhibited the greatest decline in T_3-I^{131} uptake between day 0 and 2, which probably reflects, to a certain extent, the degree of cold shock suffered by the cells in their respective treatments. The spermatozoa suspended in N-J solution cooled to 7°C and undoubtedly cold-shocked by this treatment showed the greatest decline. Apparently, the spermatozoa on this treatment also already showed the effects of cold shock at the time of the first assay, as their uptake value was relatively low even though the degree of motility exhibited was similar to that of the other treatments. Thus, in this instance, the uptake of T_3-I^{131} was more indicative of the effects of cold shock than motility. At the second assay, day 2, both measures reflected the effects of cold shock on the sperm cells.

Summary. The uptake of triiodothyronine-I^{131} by washed bull spermatozoa was found to have a positive association with sperm cell concentration and period of incubation with the labeled T_3. Spermatozoa suspended in a simple salt solution (2.9% sodium citrate) had a higher uptake of T_3-I^{131} than spermatozoa diluted in a more complex ionic medium (N-J solution). The T_3-I^{131} uptake by the sperm cells suspended in the 2 diluents increased at different rates with an increase in cell concentration. Within the range studied, addition of glucose to the 2 diluents had no effect on uptake of T_3-I^{131} by spermatozoa. Sperm cell viability greatly influenced their uptake of T_3-I^{131}. Cells killed by continuous incubation at 38°C or by treatment with acetic acid or sodium hydroxide had similar isotope uptake values which were significantly less than that of live cells. Spermatozoa killed by cold shock treatment, however, had the lowest uptake of T_3-I^{131}. Whereas, formaldehyde treatment of spermatozoa resulted in uptake values significantly greater than that of live cells. A positive association between uptake of T_3-I^{131} and motility occurred with spermatozoa stored up to 18 days in different diluents and temperatures.

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Influence of Exogenous Calcium on Calciphylaxis and Calcergy.* (30088)

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Previous studies carried out to test the calcifying potency of various substances when placed in contact with the tissues of living animals have suggested the following classification: 1) *Calcergens,* which elicit calcification in unpretreated animals(1,2,3), 2) *Calciphylactic challengers,* which induce calcification only in animals sensitized by substances capable of increasing the calcium and phosphate content of blood and inter-

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