erately with Nile blue sulfate and methyl green/pyronin G and were negative to the acid hematin test. The pigment reduced ferricyanide to ferrocyanide in Schmorl's test and was strongly positive in the indophenol and chrome-alum hematoxylin reactions. In addition, fat peroxides and traces of several amino acids were recorded. The coupled tetrazonium reaction (accompanied by its controls), and the dihydroxy-dinaphthyl-disuphide (DDD) and alkaline tetrazolium tests were moderately positive, showing the presence in the lipofuscin bodies of SH and SS groups. The pigment was also active for several enzymes, especially acid phosphatase and AS-type non-specific esterase. Our characterization of the lipofuscin pigment thus agrees with that of the aging pigment described by several workers[13-17].

The presence in the lipofuscin pigment of large amounts of lysosomal enzymes suggest that lysosomes are involved in its formation. The concept of the lysosomal origin of lipofuscin is supported by several workers who believe that the lipofuscin bodies may represent true lysosomes and be formed when lipids and lipo-protein precursors undergo peroxidation and polymerization of polyunsaturated fatty acids[18-21].

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Uptake of glucose-C^{14} and fructose-C^{14} by buffalo spermatozoa in extenders during cold storage

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Summary. Buffalo spermatozoa exhibit insignificant uptake of glucose-C^{14} and fructose-C^{14} when semen samples were preserved in cold for 96 h in 2 extenders. Incorporation of C^{14} in spermatozoa, TCA precipitable proteins and soluble sugar phosphates was either equal or less in semen samples preserved in cold. CAW appeared to be a better extender than SKMEY as revealed by sugar uptake in cold.

The survival of spermatozoa in the environment of an extender for a reasonable period during cold storage is a pre-requisite for its use in artificial insemination[2]. The conventional egg-yolk citrate (EYC) diluent used for bull semen[3] is less effective for buffalo semen preservation[4], and the buffalo spermatozoa survived for a shorter time than bull spermatozoa[4-6]. Recently, it has been reported from this laboratory[7,8] that citric acid whey (CAW) is a superior diluent for the preservation and freezing of buffalo semen. The preservation characteristics of buffalo semen in this extender has also been reported[9]. The leakage of acrosomal enzyme like hyaluronidase is relatively much less in buffalo spermatozoa preserved in CAW in cold and frozen condition[10]. In the light of these observations, the uptake of glucose-U-C^{14} and fructose-U-C^{14} by buffalo spermatozoa extended in CAW and in skim-milk egg yolk[11] (SKMEY) had been studied when preserved in cold. This report presents these data which indicate relatively less uptake of these sugars by the sperm when preserved in CAW.

Materials and methods. Ejaculated semen was collected by means of an artificial vagina[12] from 6 Murrah buffalo bulls of the institute herd. 2 successive ejaculates were

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Table 1. Incorporation of glucose-C^{14} and fructose-C^{14} into semen components during cold preservation in extender

Radioactive sugar substrate	Extender used	Storage period (h)	Specific activity in fractions			
			Spermatozoa	Proteins (TCA precipitate)	Sugar phosphates Barium soluble	Barium insoluble
Glucose-U-C^{14}	CAW	0	8.19	0.54	10.21	7.76
		96	2.55	0.55	2.35	7.62
Fructose-U-C^{14}	CAW	0	1.72	0.64	4.01	9.93
		96	1.48	0.58	2.67	11.78
Glucose-U-C^{14}	SKMEY	0	0.70	0.55	3.54	12.57
		96	0.88	0.37	2.80	5.88
Fructose-U-C^{14}	SKMEY	0	1.30	0.23	2.40	9.74
		96	0.41	0.14	2.51	7.52

Table 2. Motility of buffalo spermatozoa at 5–7 °C in extenders before and after storage

Diluent used	% Motility 0 h	96 h
CAW	85.0	58.0
SKMEY	78.0	43.0

combined for such study. Those samples with a mass activity of 3–4 or greater[12] were pooled. The split samples were diluted in the ratio of 1–10 with the extender. The extenders used were CAW and SKMEY prepared according to Ganguli et al.[7] and Tomar and Desai[11], respectively. Penicillin and streptomycin were added at the rate of 1000 IU and 1.5 mg per ml of the diluent. Semen samples after dilution were used further for the subsequent studies. Seminal plasma was obtained by centrifuging diluted semen at 10,000 rpm for 30 min under refrigerated conditions, and it was preserved in refrigerator. Glucose-U-C^{14} and fructose-U-C^{14} were purchased from the Bhabha Atomic Research Centre, Bombay.

The assay system for studying the uptake of the radioactive sugars contained 3 ml of diluted semen and 0.1 ml of C^{14} sugar having 1.0 μci. The samples were incubated for 0 and 96 h in refrigerator after which the reactions were terminated by trichloroacetic acid (TCA) after the removal of the spermatozoa by centrifugation as indicated above. The separated spermatozoa were washed 5 times with 5 ml each of normal saline solution and the associated radioactivity in the sperm was measured. The supernatant obtained was immediately treated with 2 ml of 30% TCA to precipitate the proteins. The precipitated material was washed successively with 6% TCA, boiled with 6% TCA and again washed with cold 6% TCA. The precipitate thus obtained was dissolved in 1 ml N NaOH; 0.5 ml of casein hydrolysate was then added and allowed to stand for 10 min at room temperature. The proteins from this mixture were again precipitated by 3 ml of 16% TCA and washed successively with 5 ml ethanol, ethanol-ether mixture (1:1) and ether. The dry precipitate thus obtained was then dissolved in 0.5 ml concentrated NH_4OH solution and transferred to weighed planchet for the measurement of radioactivity.

The supernatant obtained after the TCA precipitation was finally fractionated for the isolation of the sugar phosphates according to Umbreit et al.[13]. 2 major fractions, namely barium-insoluble sugar phosphates and barium-soluble-alcohol-insoluble sugar phosphates were isolated and measured for radio-activity. All radioactive measurements were done in a Geiger-Müller counter and results were expressed in sp. act. (cpm/mg) after necessary corrections for background counts and self absorptions.

The motility of spermatozoa was evaluated by the method of Salisbury and VanDemark[14], and progressive motility was taken as the assessment criterion.

Results and discussion. The spermatozoa appear to incorporate glucose-C^{14} and fructose C^{14} when preserved in both the extenders tested, namely CAW and SKMEY as evident from the data in table 1. The extent of incorporation was dependent on the period of preservation in the cold. In case of the distribution of C^{14} in spermatozoa, uptake of glucose-C^{14} was more in CAW tended semen than fructose-C^{14} at 0 °C preservation. With preservation period (96 h in 0 °C) there was a decrease in the radioactivity incorporated from glucose-C^{14} in the spermatozoa when extended in CAW compared to fructose-C^{14}. Past reports also indicate that glucose is a more important substrate than fructose[15,16]. However, glucose and fructose were observed to be utilized at about the same rate by washed spermatozoa as evaluated from $C^{14}O_2$ production[17]. It was further remarked that when the 2 sugars are presented to washed spermatozoal suspension together, glucose is utilized before fructose.

No difference was observed in the incorporation of these sugars in the protein fractions of seminal plasma when semen was preserved in cold for 96 h in CAW as the extender. Whereas in SKMEY as extender, there was some decrease in the radioactivity in protein fraction on preservation. It appears, therefore, that the sperms when in SKMEY are undergoing metabolic processes although preserved in cold.

Hexose are normally metabolized via the formation of sugar phosphates of the Embden-Meyerhof Pathway in living organisms[18]. These intermediates were classified and fractionated by Umbriet et al.[13] into 2 major groups on the basis of their solubility as barium salt. Result in table 1 show that the barium-insoluble sugar phosphates, namely fructose-1, 6-diphosphate, 3-phosphoglyceric acid do not alter in its turnover rate during cold storage spermatozoa, unlike the barium-soluble sugar phosphates, namely glucose-1-phosphate, glucose-6-phosphate, fructose-6-phosphate, etc. All these observation indicate that the spermatozoa when preserved in cold in extender are not appreciably in a dynamic state, as apparent from the low level of incorporation of radioactivity from these sugars into the different metabolites. However, in this respect, CAW appears to keep the spermatozoa at a less active stage when preserved in cold, compared to SKMEY, in relation to the utilization of glucose and fructose. This could partially explain the better storage property of CAW as an extender for buffalo semen[9], which is also supported by the motility data in table 2.

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