dilute suspension had lost CO_2 below a minimum essential level.

This study and a previous study(9) show that high dilution and the washing technique cause a loss of CO_2 resulting in reduced metabolism. CO_2 may also have been a contaminant in substances reported by others(2, 4) which prevented the decrease in motility on high dilution. For example, egg yolk has been shown to prevent the deleterious dilution effect(2) and in our laboratory egg yolk has been shown to substitute for CO_2 in the metabolism of dilute suspensions of spermatozoa (unpublished). The effect of egg yolk is believed to be due to a high solubility of CO_2 in lipid material(12).

From the results presented here it should be possible to use higher dilution rates in CO_2-containing diluters for artificial insemination than is commonly used in conventional diluents. Evidence for such a hypothesis has been reported by Foote(5) who found no significant decrease in fertility from inseminating 5×10^6 as compared to 10×10^6 live spermatozoa extended and stored in carbonated CUE diluent.

Summary. An experiment was designed to test the effect of CO_2 in preventing the deleterious dilution effect on metabolism and motility of spermatozoa in varying concentrations from the same sample. The results showed that CO_2 prevented the depressed metabolic activity and loss of motility associated with high dilution. Thus, CO_2 is one essential factor which is removed from the sperm cells upon excessive dilution.

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Effect of CO_2 on Anaerobic Glycolysis of Unwashed, Once- and Twice-Washed Spermatozoa from the Same Semen Samples. (28503)

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Detrimental effects on the motility and metabolism of mammalian spermatozoa from removal of seminal plasma by washing have been reported(4,5,10,11) and glycolysis was more markedly depressed than was respiration. The cause has been suggested as 1) removal of a necessary component for glycolysis (12) and 2) possible mechanical injury due to centrifugation(5). The detrimental effect of washing has been found to be partially but not completely prevented by including low levels of potassium in the washing solution

(12) and by including seminal plasma in the incubation diluent(5). We have shown(3) that above physiological levels of phosphate would partially restore the glycolytic ability of twice-washed spermatozoa under both aerobic (air) and anaerobic (N_2) conditions. However, under aerobic conditions the levels necessary for stimulation of glycolysis were inhibitory to respiration. Salisbury and Kinney(6) have reported that metabolic CO_2 stimulated aerobic glycolysis of diluted semen. This stimulatory effect has been repeated in more recent studies for diluted semen, but the stimulation was not so apparent for twice-washed spermatozoa(2).

The authors(3) have suggested that CO_2 might be one of the components removed by the washing process and an apparent CO_2 requirement for the anaerobic glycolysis of epididymal-like bovine spermatozoa(1) suggested that CO_2 might also be deficient in twice-washed spermatozoa which would, at least partially, explain their depressed glycolysis. This paper reports the results of a study comparing the effects of CO_2 on the anaerobic glycolysis of bull spermatozoa, unwashed, once- and twice-washed from the same samples.

Procedure. Methods of semen collection, washing, and chemical determinations have been described(5). Measurement of metabolic activity under anaerobic conditions has also been described(8). Each semen sample was divided into 3 portions, one was left as semen, another was used for obtaining once-washed cells, and the last portion was used for obtaining twice-washed cells. Sperm cells in the washed portions were washed and resuspended in physiological saline to contain approximately the same sperm cell concentration as in the semen. Semen and washed cell suspensions were diluted 1:4 in the Warburg flasks, after equilibration, in a citrate-bicarbonate diluent containing fructose (IVT) (9) and incubated for 4 hours at 37°C. Flasks were gassed with either 100% N_2, N_2 containing 5% or 25% CO_2, or 100% CO_2 by alternately gassing and evacuating with vacuum. CO_2 evolved from the reaction of metabolic acid and bicarbonate (corrected for CO_2 and acid retention), the fructose disappearing, and the lactic acid accumulating during the incubation period were measured. Motility was estimated at the end of the incubation period by direct microscopic examination after aeration of samples removed from the flasks.

Results. Spermatozoa in diluted semen showed their maximum metabolic activity when gassed with 100% N_2, and each increase in pCO_2 was progressively inhibitory (Table I), which is the same response as previously reported(7,8). Metabolic activity of once-washed spermatozoa gassed with 100% N_2 was substantially reduced from that of the unwashed controls in contrast to a slight stimulation of glycolysis by once-washed sperm cells gassed with 5% CO_2 95% N_2. The result was a stimulation of metabolism by 5% CO_2 as compared to 100% N_2 with once-washed sperm cells. As with semen, 25% and 100% CO_2 were progressively inhibitory. When spermatozoa were washed twice there was little metabolic activity by the cells subjected to 100% N_2, whereas, the cells subjected to N_2 containing 5% CO_2 exhibited normal metabolic activity. As the pCO_2 was increased to 25% and 100% CO_2 the inhibitory effect on metabolism became increasingly apparent.

Glycolytic activity was depressed by each additional washing when the flasks were gassed with 100% N_2. However, when the N_2 gas contained 5% CO_2 there was no depression of glycolysis with each successive washing; in fact there was a trend toward a higher metabolic rate by spermatozoa removed from the seminal plasma. Thus, it must be concluded that each successive washing depleted spermatozoa of CO_2 until the cells were unable to carry on glycolytic processes to supply energy for motility. This deficiency was not observed when seminal plasma was present because with mixing of the semen and the bicarbonate in the diluent enough CO_2 was evolved by reaction from the acids (in semen) and the bicarbonate to initiate and maintain glycolysis. With once-washed sperm cells even though most of the acids of semen were removed, as evidenced

TABLE I. Effect of Carbon Dioxide on Anaerobic Glycolysis in a Citrate-Bicarbonate Medium of Spermatozoa Unwashed, Once-, and Twice-Washed from the Same Semen Samples When Incubated for 4 hr at 37°C. (n = 10)

Cell type	Gas	CO_2 evolved ($\mu l/10^8$)	−Fructose ($\mu g/10^8$)	+L.A. ($\mu g/10^8$)	Final motility (%)
Semen	100%N_2	257	1718	897	44
	5%CO_2 95%N_2	158	1496	767	54
	25%CO_2 75%N_2	126	796	503	46
	100%CO_2	67	657	227	51
Once-washed	100%N_2	158	1177	574	22
	5%CO_2 95%N_2	173	1889	683	31
	25%CO_2 75%N_2	108	1102	369	30
	100%CO_2	50	508	152	33
Twice-washed	100%N_2	27	214	81	4
	5%CO_2 95%N_2	188	1640	843	29
	25%CO_2 75%N_2	135	1010	389	25
	100%CO_2	48	409	107	22

by the initial CO_2 evolved on mixing, there apparently was enough CO_2 retained by the cells to initiate glycolysis. However, the stimulation by 5% CO_2 suggests that the CO_2 level was below optimum. The twice-washed sperm cells were depleted of extracellular (from acid) and probably of intracellular CO_2 by the gassing procedure and an exogenous supply of CO_2 was required for glycolysis. The required CO_2 must be in the "free" form since bicarbonate was present in the diluent and glycolysis did not occur unless CO_2 was introduced into the gaseous atmosphere.

Motility data (Table I) suggest that energy for motility was not produced in the absence of CO_2. Motility of twice-washed spermatozoa under 100% N_2 was markedly reduced in comparison to motility where varying levels of CO_2 were present. In 5 of the 10 samples there was no motility after subjection to 100% N_2 during the incubation period. Metabolic activity of spermatozoa subjected to 100% CO_2 was not much different from that of twice-washed sperm cells under 100% N_2 but the ability to recover motility upon exposure to air suggests that the energy stores must have been preserved by CO_2 inhibition. Inability to recover motility after subjection to 100% N_2 was probably not due to a chemical inhibition of the use of energy-rich phosphate stores, but may be due to their exhaustion and failure of replenishment in the absence of sufficient CO_2 to initiate utilization of the chemical energy from the fructose substrate.

Cellular concentration of the twice-washed sperm cells may influence the response to 100% N_2 gassing since in 2 additional samples substantial metabolism occurred under N_2 and some motility was maintained during the incubation period. Mean sperm cell concentration of these 2 samples was 3.222×10^8 per flask as compared with a mean of 2.268×10^8 cells for the 10 samples which showed little or no metabolism. Metabolism by more concentrated sperm cells under 100% N_2 suggests that enough CO_2 was retained by the cells to initiate glycolysis and the CO_2 evolved was enough to allow continuation of energy production and maintenance of motility.

Summary. A minimum partial pressure of carbon dioxide is required for the glycolytic processes furnishing energy for motility of spermatozoa. The washing process for obtaining twice-washed sperm cells depletes the cells of CO_2 below the minimal required level for glycolysis and motility and must be replaced from exogenous sources when the cells are in dilute concentration.

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Maintenance of Adrenalectomized Dogs with Low Plasma Volumes and Normal Electrolyte Patterns.* (28504)

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The plasma volume and electrolytes of intact and adrenalectomized dogs can be severely reduced and the animals maintained without apparent detriment to their activity and vigor(1-4). The present experiments are concerned with a simple method for drastically modifying the fluid and salt balance of adrenalectomized dogs by contracting their plasma volumes within a matter of minutes and keeping them low for 8-12 days with little or no deviation from normal of the plasma electrolyte patterns.

Methods. The adrenalectomized dogs were strong, active animals with normal arterial pressures, plasma volumes and plasma electrolytes. They had been operated for periods of 8 months to 1 year and table-trained for blood pressure and plasma volume studies. The dogs listed in Table I were maintained on 1 mg/day of desoxycorticosterone acetate (DCA) plus salt supplements in the ration. The oil-soluble steroid was omitted the day the experiments began and i.v. injections of 1 mg/day of the solubilized free alcohol of desoxycorticosterone (DOC) substituted.† There were 2 exceptions to this regimen: Dog 4, Table I, received 2 mg/day of DOC and Dog 5 was given 1 mg/day of aldosterone (Ciba). After securing control samples, the animals were injected i.v. with 300 mg of pentylenetetrazol (Metrazol, Knoll). Blood was drawn immediately after the severe reaction subsided and the plasma volume determined, using a standardized sample of the dye T-1824 and methods recommended by Gregersen and Stewart(5) and Chien and Gregersen (6), with a slight modification as to the time interval between samples.

A control blood sample was drawn from the jugular vein using a heparinized syringe; a solution of the dye, measured gravimetrically was then injected into the vein from an accurately calibrated syringe. This was cleared of all dye by rinsing repeatedly with blood drawn in and out of the barrel. Five ml samples were collected without stasis through an indwelling needle at 10, 13, 16 and 19 minute intervals after injection. To account for any residual dye which might be retained from previous determinations, the initial sample taken before dye injection was used to set the zero on the Beckman Model B. spectrophotometer employed for the determination. The analyses were made using 1 ml of plasma, 10 mm cuvettes and read at 620 mμ.

The animals were placed in metabolism cages, allowed free access to water but the food for Dogs 1-5 was given daily by stomach tube and consisted of 120 g of Lonalac

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† Solubilized desoxycorticosterone is prepared by dissolving the free alcohol of the steroid in hot, absolute alcohol and with constant stirring slowly adding, dropwise, propylene glycol followed by water. The final solution should contain 25% alcohol, 25% propylene glycol and 50% water. Aldosterone for i.v. use is prepared in the same way.