was responsible or whether polygenic factors were at play.

In Stage 2, the trend toward resistance was not maintained, and it appears that other factors, either environmental or genetic in nature, had a modifying effect. Although environmental conditions may have been responsible for the change in Stage 2 these were not evident. No relation could be established with season, housing, diet, intercurrent disease, or personnel. Only 7 animals died of intercurrent disease during the experimental period which lasted 4 years.

Food intake and growth rates (Fig. 2) were similar for resistant and susceptible animals in Stages 1 and 2. Resistance in these animals appeared to be unrelated to rate of growth.

It is possible that as a result of inbreeding in Stage 2, modifying genes became homozygous and thus led to an increase in susceptibility. It also is possible that the decline in resistance was due in part to what is termed "inbreeding depression," although the nature of the latter is not clear.

Summary and conclusions. Male rats of the Sprague-Dawley strain, which were found to be resistant to the development of dietary cirrhosis, were bred with random females of the same strain. In 3 generations of rats so bred there appeared to be a sharp increase in the proportion of resistant males. However, when resistant males derived from resistant fathers and grandfathers were crossed with their daughters or sisters there was a sharp decline in the proportion of resistant male offspring. It is suggested that resistance to dietary cirrhosis in the rat may be attributable to an inherited trait or traits, and that other factors, either environmental or genetic, may exert a modifying influence.

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Carbon Dioxide in Prevention of the Deleterious Dilution Effect on Metabolism and Motility of Mammalian Spermatozoa. (28502)

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Mammalian spermatozoa, as well as spermatozoa from lower animals, have been shown to exhibit a dilution effect. Gray(7) reported that mild dilution of sea-urchin sperm stimulated motility and O_2 uptake, but was detrimental to livability. Salisbury *et al* (13) found that mild dilution of bull spermatozoa depressed livability, but did not adversely affect fertility. Later it was shown that mild dilution stimulated O_2 uptake of bull sperm depending upon the ions present in the diluting medium(1). The dilution effect and the proposed theories as to its cause have been reviewed(10,14). It is generally agreed that mild dilution stimulates motility and metabolism, but further dilution beyond an optimum sperm cell concentration depresses them both(2,3,4,11,17,18).

The depressive effect is believed to be caused by a) a loss of vital material from

the spermatozoa(4), b) physical changes of the sperm cells(10), and/or c) an upset in ionic exchange(10,17). The first (a) has probably received the most support and it has been reported that the addition of several substances (*i.e.*, egg yolk, cell-free supernatants from more concentrated samples, glycogen, etc.) increased the resistance of spermatozoa to the effects of high dilution(2,4). Although the mode of action has not been satisfactorily explained, these substances may prevent the loss of some vital material from the spermatozoa(3,4). However, it has also been suggested that these substances might contain a common contaminant necessary for metabolism and motility(4). Several investigators(4,10,17) have postulated that the deleterious dilution effect and the depressed metabolic activity and motility caused by excessive washing of spermatozoa have the same basic cause; the loss of vital material. Attempts to correlate a factor preventing both phenomena have been unsuccessful(17).

We have reported recently a need for CO_2 in the anaerobic metabolism of spermatozoa (8,9) in which it was observed that the need could be demonstrated only when the cellular concentration was low. However, no comparison was made of different cell concentrations from the same sample, so that some of the differences could have been due to sample variability. Thus, an experiment was designed to test the effect of CO_2 on the anaerobic metabolism of varying concentrations of spermatozoa from the same sample. This paper reports the results of that study.

Methods and materials. Epididymal-like spermatozoa were collected as previously described(6). After the spermatozoa were washed and resuspended in saline (0.9% NaCl) to give the highest concentration, portions of this sperm cell suspension were diluted with saline to give 3 additional cellular concentrations from the same sample. This procedure was followed for 4 different spermatozoan samples. Anaerobic metabolism of cells in a citrate-bicarbonate medium(16) containing fructose was studied in duplicate in a Warburg respirometer at 37°C for 4 hours. The flasks were gassed with 100% N_2 or 5% CO_2 95% N_2 by alternately gassing and evacuating with vacuum. The CO_2 evolved by the reaction of metabolic acid with bicarbonate (corrected for CO_2 and acid retention), the fructose disappearance, and lactic acid accumulation were measured. Techniques for determining CO_2 evolution, fructose and lactic acid analyses have previously been reported(15). Motility was estimated by microscopic observation after aeration of the samples.

Results. The results (Table I), as the mean of duplicate determinations, showed that with higher concentrations of sperm cells (3.9 to 8.0 $\times$ 10^8) there was little difference in total metabolism when the cells were subjected to 100% N_2 or 5% CO_2 95% N_2. However, the fructose had been completely utilized in most of these samples during the 4-hour incubation and a better comparison is the CO_2 evolved during the first hour. With the 3 highest concentrations (6.6 to 8.0 $\times$ 10^8) there was inhibition of metabolism by 5% CO_2 as compared to N_2, whereas, with concentrations of 3.9 to 5.4 $\times$ 10^8 there was a definite stimulation by 5% CO_2 in 2 of the 3 concentrations. It would appear as though enough CO_2 was retained by cell concentrations between 6.0 and 8.0 $\times$ 10^8 to create an optimum level of CO_2 for maximum metabolism since 5% CO_2 was inhibitory. With concentrations between 4.0 and 6.0 $\times$ 10^8 the CO_2 retained was apparently below optimum since in 2 of the 3 observations 5% CO_2 was stimulatory to metabolism during the first hour. Since metabolism in these 2 samples was about equal after 4 hours incubation, an optimum CO_2 level must have been reached in the N_2 atmosphere between one and 4 hours.

The effect of the gaseous atmosphere on metabolism and motility of cell concentrations ranging from 1.2 to 3.4 $\times$ 10^8 was striking. In all cases 5% CO_2 95% N_2 supported higher metabolic activity and motility of the spermatozoa. Results from cell concentrations between 2.2 and 3.4 $\times$ 10^8 gassed initially with 100% N_2 were variable in that the metabolic activity ranged from essentially zero to almost normal as compared with an

TABLE I. Effect of Cell Concentration and CO_2 on Initiation of Anaerobic Metabolism of Epididymal-like Bovine Spermatozoa.

	100% N_2				5% CO_2 95% N_2			
Sperm conc. $\times 10^8$	CO_2 evolved 1 hr ($\mu l/10^8$)	CO_2 evolved 4 hr ($\mu l/10^8$)	−Fruct. ($\mu g/10^8$)	+L.A. ($\mu g/10^8$)	Final motility (%)	CO_2 evolved 1 hr ($\mu l/10^8$)	CO_2 evolved 4 hr ($\mu l/10^8$)	−Fruct. ($\mu g/10^8$)	+L.A. ($\mu g/10^8$)	Final motility (%)
[c]7.976	87	91	369*	286	30	65	75	369*	284	40
[d]6.794	109	125	433*	312	30	79	91	433*	295	35
[b]6.594	105	117	467*	332	45	84	86	467*	340	40
[a]5.390	32	99	439	347	25	72	108	573*	406	55
[c]5 390	68	138	546*	415	30	67	114	546*	420	25
[d]3.878	22	167	697*	505	30	73	154	648*	489	25
[b]3.376	12	12	44	32	0	80	151	804	580	50
[a]3.106	36	114	447	313	20	84	169	844	600	60
[c]3.016	17	63	231	176	0	72	195	975*	709	10
[d]2.926	5	10	73	4	0	78	192	953	695	40
[b]2.224	16	24	116	108	0	114	223	1248	962	50
[c]2.084	1	7	187	0	0	26	141	556	438	20
[a]2.014	8	15	2	35	5	18	29	35	79	50
[b]1.684	0	−3	38	0	0	102	255	1253	954	40
[a]1.192	6	18	29	13	3	23	49	394	310	40
[d]1.192	−5	3	216	0	0	53	256	904	929	30

* All fructose was utilized.

[a, b, c, d] different samples.

apparently normal metabolic activity of cells from the same sample under 5% CO_2. Even though some metabolic activity was observed in some samples in this cell concentration range, the increased survival of sperm cells under 5% CO_2 was readily apparent from the motility estimates after incubation.

Cell concentrations below 2.1×10^8 and gassed with 100% N_2 essentially did not metabolize during the 4-hour incubation and little motility was observed at the end of the incubation period. In contrast, good motility was observed in the samples subjected to 5% CO_2 and, with the exception of one sample, metabolism was also substantial. Thus, it must be concluded that CO_2 is an important factor for glycolysis and maintenance of motility of spermatozoa in dilute suspension.

Discussion. The results from this study confirm the speculation of the previous reports(8,9) that CO_2 is required for normal metabolism of spermatozoa in dilute suspension and that control of the cellular concentration is necessary to demonstrate the need. It would appear from the results in our laboratory that twice-washed sperm cells resuspended in saline and diluted in a citrate-bicarbonate medium to cell concentrations above 4.0×10^8 retain enough CO_2 to initiate glycolysis and maintain motility. Whereas, sperm cells from the same sample diluted to contain less than 2.0×10^8 will not metabolize nor maintain motility unless an exogenous source of free CO_2 is supplied. The response of sperm cells to concentrations between 2.0 and 4.0×10^8 is variable and not predictable. Some samples will metabolize and some will not when gassed with 100% N_2.

A Fisher gas partitioner was used in an attempt to determine the level of CO_2 retention of varying concentrations of sperm cells after gassing with 100% N_2. The results obtained for the pCO_2 in the liquid and gas phases were not conclusive because of the time lapse between sampling and analysis. However, the results suggested that enough CO_2 was retained by the more concentrated cells to initiate metabolism and the cells in

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