SUMMARY

1. Sugars present in nectar secreted by twelve species were separated on paper chromatograms, and quantitative determinations of the amounts of glucose and fructose were made. The total sugar content of each sample was measured with a refractometer.

2. The proportions of glucose and fructose varied greatly in nectar from different species.

3. For any one species, the proportions of the different sugars appeared to remain relatively constant.

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The Metabolism of Pyruvate in Bull Spermatozoa

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Although the metabolism of semen is considered to be predominantly glycolytic (see Mann, 1949), many studies of its respiratory metabolism have been made, some with a view to finding a correlation between respiratory activity and fertilizing capacity (Walton & Edwards, 1938; Gosh, Casida & Lardy, 1949). In such studies much attention was given to the magnitude of the endogenous respiration of washed spermatozoa, and where the metabolism of added substrates was studied conclusions regarding their fate were in most cases drawn from respiration measurements. In the present paper an investigation into the metabolism of pyruvate in bull spermatozoa is reported. It was suggested by observations made in a previous study (Terner, 1951) that in an actively synthesizing tissue, such as the lactating mammary gland, pyruvate is incompletely oxidized and that its complete oxidation is brought about by 2:4-dinitrophenol (DNP). Since the latter is known to inhibit synthetic reactions

(see Clifton, 1946, 1952) it was believed that the magnitude of the effect of DNP on the respiration of spermatozoa metabolizing pyruvate might provide a measure of their metabolic vigour based on their synthetic activity. The results of the studies now reported suggest that it may be possible to assess the quality of spermatozoa by manometric measurements under certain experimental conditions. A preliminary account of the work described in this paper has been published (Melrose & Terner, 1951, 1952).

EXPERIMENTAL

Preparation of sperm suspensions. The semen of bulls of various dairy breeds, kept for the purpose of artificial insemination, was collected by the usual short pattern artificial vagina (Walton, 1945). All ejaculates were examined immediately after collection for motility and sperm density. For the purpose of this paper no attempt was made to distinguish between grades of motility other than 'good' and 'poor'. Samples were considered to be of good motility

when they showed 80% motile spermatozoa and an active wave motion. Poor samples had a high percentage of immotile spermatozoa and the wave motion, if present, was sluggish. Only samples of good motility were selected for manometric experiments and generally two or three ejaculates from the same bull were combined if satisfactory. The initial work was confined to bulls of known high fertility, but subsequently samples from bulls of lower fertility were also used. In most experiments the spermatozoa were separated from the seminal plasma by centrifugation and washing as described by Tosic & Walton (1950). The washed suspensions were again examined for motility and sperm density. Only samples of good motility were used. The final dilution of the resuspended spermatozoa or of whole semen was adjusted so as to give a sperm count of $2-4 \times 10^8$ spermatozoa/ml. final suspension. In order to avoid differences in the metabolism of pyruvate between different sperm suspensions, due to the possible presence or absence of intermediates of the citric acid cycle, 0.4 ml. of 0.2 M-fumarate was routinely added for every 30 ml. of the final sperm suspension when required for aerobic experiments. The density of the sperm suspensions before and after the final dilution was determined by the Thoma haemocytometer slide method.

Suspension media. Phosphate saline without Ca (Krebs & Eggleston, 1940) was the standard medium. In some experiments the composition of this saline was altered by replacing the phosphate buffer by citrate (0.066 M, pH 7.3). This saline will be referred to as citrate saline. In some anaerobic experiments the spermatozoa were suspended (in about half the volume required for the final suspension) in a modified saline which contained one-third of the amount of the phosphate buffer of the Krebs-Eggleston phosphate saline. The suspension was then diluted to its final strength by adding 0.154 M- NaHCO_3 and Krebs-Eggleston saline (without added phosphate buffer). For every 30 ml. final suspension, 6.2 ml. 0.154 M- NaHCO_3 were added. This medium will be referred to as low-phosphate-bicarbonate saline.

Manometric measurements. Portions (3.0 ml.) of the sperm suspension were pipetted into conical Warburg vessels of approx. 25 ml. capacity. Substrates and inhibitors were placed in the side arms of the vessels and 0.154 M- NaCl was added to make the total volume up to 4.0 ml. When whole semen of low fructose content was used, fructose was added to the diluted suspensions in amounts calculated to ensure an initial concentration of 0.0025–0.005 M in the Warburg vessel. When the O_2 uptake was measured the gas phase was air, and CO_2 was absorbed by alkali. In anaerobic experiments the gas phase was either 100% N_2 or a mixture of 5% CO_2 + 95% N_2 (v/v). Strictly anaerobic conditions were maintained by a stick of yellow phosphorus in the centre well. The substrates and inhibitors were mixed with the sperm suspensions either immediately before the vessels were placed in the thermostat at 37°, or after 10–15 min. equilibration. In the latter case, the incubation periods given in the tables do not include the equilibration periods before mixing. After incubation, each vessel was placed in iced water and 1 ml. 2 N-HCl was added. Zero-time control flasks underwent the same treatment as the experimental vessels up to the moment of the start of the incubation period as defined above.

Analytical methods. Pyruvate was estimated by the method of Friedemann & Haugen (1943) after deproteinization with metaphosphoric acid; lactic acid was determined

by the method of Barker & Summerson (1941) after deproteinization with trichloroacetic acid. Fructose was determined according to Roe (1934) or as reducing substance by the method of Nelson (1944). When the changes in fructose content of the samples were large the two methods gave essentially identical results.

Metabolic quotients. All metabolic quotients are expressed in the Z notation ($\mu\text{l.}/10^8$ spermatozoa/hr.) (Redenz, 1933); $Z_{\text{lactic acid}}$ denotes the rate of lactic acid production or utilization as determined by chemical methods, while Z_{CO_2} applies to rates of CO_2 evolution calculated from manometric pressure changes.

Conception rates and fertility of bulls. In accordance with the usual practice, conception rates were calculated on the 'non-return' basis, i.e. the percentage of cows and heifers not reported to have required a repeat insemination within a period of 16 weeks from the date of the first insemination. The average fertility of each bull was assessed from its breeding records for the preceding 12 months and expressed as percentage non-returns.

RESULTS

Aerobic metabolism of pyruvate in spermatozoa. In agreement with the findings of Lardy & Phillips (1943c), it was observed that the respiration of washed bull spermatozoa was increased by the addition of pyruvate. However, their observation that DNP (10^{-4} M) further stimulated the respiration could only be confirmed in samples from certain bulls. In many cases DNP had no effect or caused some inhibition of oxygen consumption in the presence of pyruvate, although it usually stimulated respiration in the presence of fructose (Table 1). Investigation of the fate of pyruvate by chemical analysis revealed that pyruvate was in part converted to lactate (Table 2). Accordingly, the amount of pyruvate metabolized by oxidative reactions is given by the difference between pyruvate disappearing and lactate formed, and will be denoted as 'net pyruvate' (Olson, Pearson, Miller & Stare, 1948). DNP was found to abolish the conversion of pyruvate to lactate and to inhibit the rate of oxidative disappearance of pyruvate ($Z_{\text{net pyruvate}}$). In most experiments DNP increased the ratio O_2 consumed/net pyruvate, even in those samples in which it did not increase the respiratory rate. Although in some cases the ratio O_2 /net pyruvate was within the theoretical limits (less than 2.5), it was often found to be greater (Tables 2 and 3). This suggested that the endogenous respiration was incompletely suppressed by the presence of the added substrate.

Endogenous respiration and effect of DNP. Lardy & Phillips (1943a) reported $-Z_{\text{O}_2}$ values of 16–30 for the endogenous respiration of washed bull spermatozoa. In a later study, however, Gosh *et al.* (1949) found what they considered exceptionally low values ($-Z_{\text{O}_2} = 3-10$). In the present investigation endogenous $-Z_{\text{O}_2}$ values in the range 6–28 were observed. The magnitude of the endogenous

Table 1. *Effect of pyruvate, fructose and 2:4-dinitrophenol on respiration of spermatozoa*

(Washed bull spermatozoa incubated in Warburg vessels at 37°. S1, G8 and F1 in phosphate saline, S12 in citrate saline; gas, air; fumarate, 0.002M, in all vessels; pyruvate, 0.005M; fructose, 0.005M; DNP, 10⁻⁴M. Z units, $\mu\text{l./10}^8$ spermatozoa/hr.)

Exp. no.	...	...	...	1	2	3	4	5	6	7
Bull	...	...	...	S1	S1	S1	S12	G8	G8	F1
Additions				-Z _{O₂}						
None				4.1	6.2	6.8	7.4	20.0	28.2	5.6
DNP				4.3	5.5	6.6	—	11.8	16.3	4.0
Pyruvate				10.8	8.6	13.9	12.8	28.5	31.4	7.7
Pyruvate, DNP				14.9	13.0	17.8	17.6	21.8	26.0	8.2
Fructose				7.9	6.9	10.6	9.6	23.8	25.0	7.7
Fructose, DNP				17.1	15.0	21.0	16.4	32.4	33.6	6.4
Sperm count $\times 10^{-8}$ /vessel				9.76	7.30	8.58	5.88	10.58	5.80	4.77
Incubation period (min.)				115	120	85	100	60	95	120

Table 2. *Effect of 2:4-dinitrophenol on metabolism of pyruvate in spermatozoa*

(Washed spermatozoa incubated in Warburg vessels in citrate saline at 37°; gas, air; fumarate, 0.002M, in all vessels; pyruvate, 0.01M; DNP, 10⁻⁴M. Incubation period, 70 min. Sperm count, 15.7 $\times 10^8$ /vessel. Bull S1. Z units, $\mu\text{l./10}^8$ spermatozoa/hr.)

Additions	-Z _{O₂}	-Z _{pyruvate}	Z _{lactate}	-Z _{net pyruvate}	Ratio Z _{O₂} /Z _{net pyruvate}
None	8.1	—	0	—	—
None	7.7	—	0	—	—
Pyruvate	13.4	39.8	29.8	10.0	1.3
Pyruvate	12.6	38.6	28.0	10.6	1.2
Pyruvate, DNP	20.0	9.4	2.7	6.7	3.0
Pyruvate, DNP	19.7	9.3	2.7	6.6	3.0

Table 3. *Effect of fluoride and 2:4-dinitrophenol on metabolism of spermatozoa*

(Washed spermatozoa incubated in phosphate saline at 37°; gas, air; fumarate, 0.002M, in all vessels; pyruvate, 0.01M in Exp. 1; 0.005M in Exp. 2; DNP, 10⁻⁴M; fluoride, 0.02M; endogenous lactate at zero time, 1.1 $\times 10^{-3}$ M in Exp. 1; 1.25 $\times 10^{-3}$ M in Exp. 2. Z units, $\mu\text{l./10}^8$ spermatozoa/hr.)

Exp. no.	Bull	Incubation period (min.)	Sperm count/vessel $\times 10^{-8}$	Additions	-Z _{O₂}	-Z _{pyruvate}	Z _{lactate}	-Z _{net pyruvate}	Ratio Z _{O₂} /Z _{net pyruvate}
1	F5	45	9.7	None	18.1	—	-6.5	—	—
				Pyruvate	25.9	45.6	+24.0	21.6	1.2
				Pyruvate, DNP	25.8	8.0	+0.6	7.4	3.5
				Fluoride	4.2	—	-5.4	—	—
				Fluoride, pyruvate	6.0	31.8	+24.0	7.8	0.8
				Fluoride, pyruvate, DNP	11.3	7.4	+1.9	5.5	2.1
2	F4	110	7.92	None	11.7	—	-4.3	—	—
				Pyruvate	21.0	30.8	+14.6	16.2	1.3
				Pyruvate, DNP	19.2	6.1	-0.4	6.5	3.0
				Fluoride	4.3	—	-2.6	—	—
				Fluoride	4.0	—	-2.3	—	—
				Fluoride, pyruvate	5.1	24.7	+16.0	8.7	0.6
				Fluoride, pyruvate	5.2	25.8	+16.5	9.3	0.6
				Fluoride, pyruvate, DNP	11.4	7.4	+2.3	5.1	2.2
				Fluoride, pyruvate, DNP	12.1	8.0	+2.5	5.5	2.2

respiration appeared to be relatively constant in a number of samples collected from the same bull at different times. Lardy & Phillips (1943c) found that DNP depressed the endogenous respiration. In the present experiments the endogenous respiration was depressed by DNP only when it was high, but was almost unaffected when it was low (Table 1).

Effect of fluoride on metabolism of spermatozoa. Fluoride, which had been used to depress the endogenous respiration of mammary gland slices (Turner, 1951), was found to be equally effective in spermatozoa. Although the rate of pyruvate utilization (Z_{pyruvate}) was decreased by 0.02M-fluoride, $Z_{\text{lactic acid}}$ was not affected. Fluoride

therefore had an inhibitory effect on the rate of oxidative removal of pyruvate ($Z_{\text{act pyruvate}}$). The respiratory rate in the presence of pyruvate was also depressed by fluoride, but DNP now caused stimulation of respiration also in those samples in which, without fluoride, it was inhibitory or without effect (Table 3).

In a study of the effects of inhibitors on the metabolism of spermatozoa, Lardy & Phillips (1943b) observed that 0.01M-fluoride caused almost complete inhibition of the endogenous respiration ($-Z_{O_2} < 1$). In the present experiments the level of endogenous respiration in the presence of 0.02M-fluoride was much higher ($-Z_{O_2} = 4-10$). This discrepancy is possibly due to different techniques in the use of fluoride. In all experiments reported in this paper fluoride was placed in the side arm of the Warburg vessel and its addition to the spermatozoa was delayed until zero time (see Potter, 1947).

Metabolism of lactic acid in spermatozoa. Lardy & Phillips (1943c, 1945) observed that DNP stimulated the respiration of washed spermatozoa in the presence of lactate, and that, in the presence of lactate alone, the respiration was lower than the endogenous respiration. Table 4 shows that although

Table 4. *Effect of 2:4-dinitrophenol on metabolism of lactate in bull spermatozoa*

(Washed spermatozoa incubated in phosphate saline at 37°; gas, air. Fumarate 0.002M and fluoride 0.02M in all vessels DNP, 10^{-4} M. Sperm count, 6.44×10^8 /vessel. Incubation period, 70 min. Endogenous lactate at zero time, 5×10^{-4} M. Bull F4. Z units, $\mu\text{l.}/10^8$ spermatozoa/hr.)

DL-Lactate added (M)	$-Z_{O_2}$	
	Without DNP	With DNP
Nil	6.4	5.1
0.00125	5.8	12.0
0.0025	6.2	14.1
0.005	10.6	18.7

lactate in low concentrations depressed the endogenous $-Z_{O_2}$ in the presence of fluoride, higher concentrations increased the oxygen consumption. DNP did not stimulate the respiration in the presence of very low concentrations of lactate, but its effect increased with increasing concentrations of lactic acid. Since only L-lactate is metabolized by spermatozoa (Lardy, Hansen & Phillips, 1945) the minimum concentration of L-lactic acid which caused increased respiration of spermatozoa in the presence of fluoride was between 0.002 and 0.003M, while DNP produced stimulation of respiration in the presence of L-lactic acid in a concentration as low as 0.001M. Chemical estimations showed that even smaller amounts of lactic acid (approx. 5×10^{-4} M), which were often present in suspensions of washed spermatozoa, were metabolized during

incubation in the absence of added substrates. Their oxidation may therefore have contributed to the 'endogenous' respiration (see Discussion). On the other hand, well-washed sperm suspensions, containing only traces of lactic acid which were not utilized during incubation, sometimes respired at relatively high rates ($-Z_{O_2}$ up to 10) in the presence of fluoride. When lactic acid was present as the only substrate in sufficiently high concentration (0.005M), its disappearance was accelerated by DNP (Table 9, Exp. 1).

Aerobic glycolysis in spermatozoa. Fructose, which has been shown by Mann (1946) to be the carbohydrate present in seminal plasma, was added to washed spermatozoa when the breakdown of carbohydrate was studied. Although in the presence of fructose $-Z_{O_2}$ was generally somewhat lower than in the presence of pyruvate, DNP always, except in some samples of poor respiratory activity, accelerated the rates of respiration, fructose disappearance and lactic acid formation (Tables 1 and 6; see also Lardy & Phillips, 1943c). When pyruvate was added to spermatozoa metabolizing fructose it rarely increased, and often decreased $-Z_{O_2}$, and had no effect on the rate of disappearance of fructose. Z_{lactate} was, however, increased, the extra lactate apparently being derived from the added pyruvate. The acceleration by DNP in the presence of fructose alone of the rates of respiration and fructose breakdown was often suppressed by added pyruvate (Table 5).

In experiments in which fluoride was added to make the rate of pyruvate formation a factor limiting the rate of glycolysis, the addition of pyruvate did not restore the reduced rate of fructose breakdown, although the rate of lactic acid formation was increased (Tables 5-7). In the presence of 0.01M-fluoride, the extra lactate formed corresponded to the amount of pyruvate disappearing (Table 5, vessels 4 and 5; Table 6, vessels 6 and 7). More lactate was formed from pyruvate + fructose in the presence of 0.01M-fluoride than from pyruvate alone (Table 6, vessels 2 and 7). It thus appears that, when the formation of pyruvate by glycolytic reactions is inhibited, added pyruvate takes part in the oxidation-reduction reaction of glycolysis. When however, glycolysis is almost completely abolished by 0.02M-fluoride, added pyruvate is metabolized as in the absence of carbohydrate (Table 7, vessels 4 and 6). On the other hand, the inhibition by DNP of the formation of lactic acid from pyruvate appeared to be partly reversed when fructose was present (Table 6, vessels 3 and 8; Table 7, vessels 5 and 7). Since Z_{pyruvate} was not increased, the extra lactate must have been derived from fructose via the oxidation-reduction step of glycolysis (Tables 5-7). Similar observations were made under anaerobic conditions (Table 7, vessels 8 and 9).

Table 5. *Effect of pyruvate and DNP on aerobic metabolism of whole semen*

(Whole semen diluted with phosphate saline incubated at 37°; gas, air. Fructose at zero time (0.0025–0.005M) and fumarate (0.002M) in all vessels; pyruvate, 0.005M; DNP, 10⁻⁴M; fluoride, 0.01M. Incubation period: Exp. 1, 80 min.; Exps. 2 and 3, 60 min. Sperm count: Exp. 1, 7.26 × 10⁸; Exp. 2, 10.18 × 10⁸; Exp. 3, 8.36 × 10⁸/vessel. Bull, F5. Z units, μl./10⁸ spermatozoa/hr.)

Exp. no.	Vessel no.	Additions	-Z _{O₂}	-Z _{fructose}	-Z _{pyruvate}	Z _{lactate}
1	1	None	15.0	20.8	—	29.8
	2	Pyruvate	18.8	20.6	16.6	36.5
	3	Pyruvate, DNP	14.5	32.0	12.7	52.4
	4	Fluoride	9.9	5.0	—	0.4
	5	Fluoride, pyruvate	8.5	8.9	12.7	14.3
	6	Fluoride, pyruvate, DNP	8.2	9.4	10.2	17.2
2	1	None	28.2	15.4	—	25.6
	2	Pyruvate	25.6	15.2	33.7	47.8
	3	Pyruvate, DNP	32.3	15.2	15.6	36.5
3	1	None	33.0	14.1	—	—
	2	Pyruvate	15.3	14.4	25.0	—
	3	Pyruvate, DNP	21.0	16.3	10.5	—

Table 6. *Metabolism of fructose and pyruvate in bull spermatozoa*

(Washed spermatozoa incubated in phosphate saline at 37°; gas, air in vessels 1–8; N₂ in vessel 4. Fumarate (0.002M) in all vessels. Pyruvate, 0.005M; fructose, 0.005M; DNP, 10⁻⁴M; fluoride, 0.01M. Incubation period, 110 min. Sperm count, 7.5 × 10⁸/vessel. Bull, G8. Fructose estimated by method of Nelson (1944). Z units, μl./10⁸ spermatozoa/hr.)

Vessel no.	Additions	-Z _{O₂}	-Z _{fructose}	-Z _{pyruvate}	Z _{lactate}
1	None	10.0	—	—	0
2	Pyruvate	31.4	—	33.0	19.8
3	Pyruvate, DNP	24.5	—	9.2	1.5
4	Fructose	26.5	31.2	—	41.4
5	Fructose, DNP	40.5	37.6	—	52.6
6	Fructose, fluoride	21.6	17.4	—	8.2
7	Fructose, pyruvate, fluoride	23.0	18.0	33.0	41.6
8	Fructose, pyruvate, fluoride, DNP	20.8	20.9	8.0	28.2
9	Fructose (anaerobic)	—	37.5	—	65.5

Table 7. *Effect of pyruvate and fluoride on glycolysis in spermatozoa*

(Washed spermatozoa incubated at 37°. Fumarate (0.002M) and fluoride (0.02M) in all vessels; pyruvate, 0.01M; fructose, 0.005M; DNP, 10⁻⁴M. Aerobic exp., phosphate saline; gas, air. Anaerobic exp., low phosphate-bicarbonate saline; gas, 5% CO₂ + 95% N₂. Sperm count, 7.2 × 10⁸/vessel. Incubation period, 100 min. Endogenous lactate at zero time, 5.9 × 10⁻⁴M. Fructose estimated by method of Nelson (1944). Z_{CO₂} not corrected for retention of CO₂. Bull, G8. Z units, μl./10⁸ spermatozoa/hr.)

Vessel no.	Additions	Experimental conditions	-Z _{O₂}	Z _{CO₂}	-Z _{fructose}	-Z _{pyruvate}	Z _{lactate}
1	None	Aerobic	7.7	—	—	—	0
2	Fructose	Aerobic	7.5	—	0	—	0
3	Fructose, DNP	Aerobic	10.9	—	2.0	—	3.6
4	Pyruvate	Aerobic	11.9	—	—	45.4	36.1
5	Pyruvate, DNP	Aerobic	20.8	—	—	13.3	3.3
6	Fructose, pyruvate	Aerobic	13.9	—	4.3	42.0	36.0
7	Fructose, pyruvate, DNP	Aerobic	16.3	—	5.0	13.3	11.0
8	Pyruvate	Anaerobic	—	12.4	—	23.8	13.3
9	Fructose, pyruvate	Anaerobic	—	20.0	5.0	28.1	24.2

Anaerobic metabolism of pyruvate in spermatozoa.

In order to investigate the mechanism of the formation of lactate from pyruvate, washed spermatozoa were incubated with pyruvate and fluoride under strictly anaerobic conditions. It was found that approximately 1 mole of carbon dioxide and 1 mole of lactate were produced from 2 moles of pyruvate.

This suggests that pyruvate undergoes dismutation according to the equation

a reaction which has been shown to occur under anaerobic conditions in animal tissues and in

Table 8. *Aerobic and anaerobic metabolism of pyruvate in bull spermatozoa*

(Washed spermatozoa incubated in low phosphate-bicarbonate saline at 37°; gas, 5% CO₂ + 95% O₂ or 5% CO₂ + 95% N₂. Fluoride, 0.02M, added to all flasks. Pyruvate, 0.01M; DNP, 10⁻⁴M. Incubation period, 80 min. Sperm count, 8.0 × 10⁸/vessel. $Z_{CO_2}^N$ not corrected for retention of CO₂. Bull, F4. Z units, μ l./10⁸ spermatozoa/hr.)

Additions	Experimental conditions	$Z_{CO_2}^N$	-Z _{pyruvate}	Z _{lactate}
None	Aerobic	—	—	—
Pyruvate	Aerobic	—	19.7	11.0
Pyruvate, DNP	Aerobic	—	9.5	4.8
None	Anaerobic	2.1	—	0.3
Pyruvate	Anaerobic	16.8	40.3	23.0
Pyruvate, DNP	Anaerobic	5.3	5.9	6.0

Table 9. *Metabolism of spermatozoa in the presence of fluoride*

(Washed spermatozoa incubated in phosphate saline at 37°; gas, air; fumarate, 0.002M and fluoride, 0.02M in all vessels; pyruvate, 0.01M; DNP, 10⁻⁴M. Endogenous lactate at zero time, 1.6 × 10⁻⁴M. Z units, μ l./10⁸ spermatozoa/hr.)

Exp. no.	Bull	Incubation period (min.)	Sperm count/vessel × 10 ⁻⁸	Additions	-Z _{O₂}	-Z _{pyruvate}	Z _{lactate}	-Z _{net pyruvate}	Ratio O ₂ /substrate oxidized
1	G2	90	10.2	None	8.6	—	-1.7	—	—
				Pyruvate	14.1	30.5	+23.4	7.1	2.0
				Pyruvate, DNP	16.0	12.5	+4.4	8.1	2.0
				DL-Lactate (0.005M)	8.3	—	-3.0	—	2.8
				DL-Lactate (0.005M), DNP	17.9	—	-5.9	—	3.0
2	G2	90	8.04	None	5.2	—	0	—	—
				Pyruvate	14.1	17.8	+11.5	6.3	2.2
				Pyruvate, DNP	13.9	10.0	+3.8	6.2	2.2
3	G2	120	9.1	None	6.0	—	-1.7	—	—
				Pyruvate	9.1	12.8	+9.0	3.8	2.4
				Pyruvate, DNP	8.2	7.0	+3.6	3.4	2.4
4	S10	110	8.64	None	7.0	—	+0.2	—	—
				Pyruvate	16.3	31.2	+24.0	7.2	2.3
				Pyruvate, DNP	20.4	13.2	+4.8	8.4	2.4
5	G8	90	11.7	None	4.9	—	-1.7	—	—
				Pyruvate	11.5	38.7	+29.6	9.1	1.3
				Pyruvate, DNP	14.9	7.4	+1.2	6.2	2.2
6	G8	100	7.2	None	7.7	0	—	—	—
				Pyruvate	11.9	45.4	+37.1	8.3	1.4
				Pyruvate, DNP	20.8	13.3	+4.3	9.0	2.3

bacteria (Krebs & Johnson, 1937; Krebs, 1937). Both the production of carbon dioxide and the formation of lactate were strongly inhibited by DNP (Table 8). An inhibitory effect of DNP on this reaction does not appear to have been reported previously. It seems probable that the formation of lactate from pyruvate under aerobic conditions is also due largely to the dismutation of pyruvate. Since, under aerobic conditions, $Z_{lactic\ acid}$ was often greater than one-half $Z_{pyruvate}$, other reactions resulting in the reduction of pyruvate may also be involved.

Effect of substrates and inhibitors on motility. The findings of Lardy, Winchester & Phillips (1945) that the motility of ram spermatozoa was maintained during incubation with pyruvate and DNP were confirmed for bull sperm. It was further observed that the spermatozoa were motile after incubation with fluoride in the presence of pyruvate or pyruvate + DNP, although in the presence of fluoride alone their motility was greatly diminished.

Endogenous respiration and fertility. Previous attempts to assess the fertilizing capacity of bull spermatozoa on the basis of their endogenous respiration have not been successful (see, for example, Gosh *et al.* 1949). No correlation between endogenous respiration and fertility was found in the present study.

Metabolism of pyruvate and semen quality. When spermatozoa of good motility, obtained from bulls of high and of low fertility, were incubated in the presence of fluoride, certain differences in their respiratory response to pyruvate and to DNP were observed. Four groups could be distinguished:

A. The respiratory rate in the presence of pyruvate was low, often only very little above the endogenous level; DNP caused large increases in oxygen consumption, often exceeding 100%.

B. The rate of respiration in the presence of pyruvate was about twice the endogenous level or higher, and DNP caused little or no further increase.

C. The respiratory rate in the presence of pyruvate was intermediate between the endogenous level and the level in the presence of pyruvate + DNP.

D. Both pyruvate and pyruvate + DNP caused only little stimulation of respiration. In a few experiments the endogenous respiration was exceptionally low and the addition of pyruvate and of DNP was without effect.

Fig. 1. Respiratory response of spermatozoa to pyruvate and DNP in presence of fluoride. Experimental conditions as in Table 9. Respiration in presence of pyruvate (A, B, C) shown in relation to endogenous respiration and respiration in presence of pyruvate + DNP (diagrammatic). A, good; B, poor; C, intermediate effect.

In a number of experiments representative of each group the changes in pyruvate and lactate content of the samples were determined. The results showed that spermatozoa of type A metabolized pyruvate with a low O_2 /net pyruvate ratio (<1), which was, however, increased by DNP to 2.0–2.5 (Table 3). Samples of type A were of excellent initial motility and their donors had exceptionally good fertility records. Samples of type B were shown to oxidize pyruvate with high O_2 /net pyruvate ratios, i.e. to completion, even in the absence of DNP (Table 9, Exps. 1, 2 and 4).

Although those samples were of good motility, the fertility of their donors was known to be poor. Spermatozoa of type C showed intermediate O_2 /net pyruvate ratios (Table 9, Exps. 5 and 6). In samples of type D pyruvate was metabolized at slow rates and with high O_2 /net pyruvate ratios (Table 9, Exp. 3).

Samples of semen from all bulls in our Artificial Insemination Centre, the breeding records of which were known, were therefore subjected to the manometric test. The oxygen consumption was followed for 60–120 min. during which time the rates of respiration were almost linear. Fig. 1 shows diagrammatically the three principal types of respiratory response.

The summarized results of respiration measurements of samples of semen tabulated against the breeding records of the donors are shown in Table 10. Dividing lines were chosen corresponding to the fertility ratings of 65 and 55 % so as to set apart the groups of bulls with outstanding breeding records and those of exceptionally poor fertility. In these groups a good correlation is shown between the results of the manometric tests and the fertility level of the bulls. The relationship is not so clearly shown in the intermediate group; this may be due to the fact that the transition of groups A and B is gradual and not easily defined, so that some samples of the extreme groups may have been included in group C.

DISCUSSION

Endogenous and substrate respiration. It has been pointed out by Mann (1949) that 'before the respiration of washed sperm suspensions can be declared a wholly endogenous process, proof must be offered first that the washing procedure has removed from ejaculated spermatozoa all extraneous material such as, for example, fructose'. In fact, in some experiments with washed spermatozoa traces of fructose could be detected in the zero-time control samples and in those cases small amounts of lactic acid sometimes accumulated during the incubation period. Nevertheless, it is doubtful whether the oxidation of fructose when present in very low concentration contributes materially to the respira-

Table 10. *Metabolic response of bull spermatozoa in relation to the fertility of the donors*

(At least three samples of semen from each bull were tested, except in four cases.)

Average fertility of bulls (expressed as % non-returns at 16 weeks after insemination)	No. of bulls	No. of samples tested	Type of metabolic response		
			Good (A)	Intermediate (C)	Poor (B, D)
Over 65	3	11	9	2	0
55–65	8	22	7	8	7
Less than 55	5	27	4	2	21
	16	60	20	12	28

$$\chi^2 = 27.4 \text{ (4 d.f.) } P < 0.001.$$

tion. It has been found by Lardy & Phillips (1943c), and confirmed in the present work, that DNP inhibits the endogenous respiration but stimulates the respiration in the presence of carbohydrate (Table 1). It thus appears unlikely that a high endogenous respiration which is inhibited by DNP can be attributed to the incomplete removal of fructose.

In the presence of fluoride the endogenous respiration was much reduced and the question arises whether the residual respiration was due wholly or partly to the oxidation of small amounts of lactate which were usually present. When the endogenous respiration was higher than the oxygen uptake calculated for the complete oxidation of the lactate disappearing, the respiration must have been partly supported by the oxidation of other endogenous substrates. When, however, fluoride was added, the residual respiration was often insufficient to allow for the complete oxidation of the lactate disappearing. This is illustrated by one experiment in which some lactate had remained in the washed sample (Table 3, Exp. 1); the addition of fluoride reduced the endogenous $-Z_{O_2}$ by about 80 %, while $-Z_{\text{lactic acid}}$ was almost unaltered. If it is assumed that the same amount of oxygen is utilized for the oxidation of equal amounts of lactate in the presence and absence of fluoride, then the low $Z_{O_2}/Z_{\text{lactate}}$ ratio in the presence of fluoride suggests that lactate was incompletely oxidized and that the high respiration in the absence of fluoride was largely due to the utilization of other substrates besides lactate. However, the possibility that in the absence of fluoride the breakdown of small amounts of fructose to lactic acid may reduce the apparent rate of disappearance of lactic acid cannot be entirely ruled out. On the other hand, the respiration of washed spermatozoa in the presence of fluoride was sometimes relatively high when only traces of lactic acid, not utilized during incubation, were present. In those cases the endogenous respiration must be attributed to other endogenous substrates. Since lactate, when added in relatively low concentration, is metabolized without causing an increased oxygen consumption, it must be concluded that the utilization of the added substrate suppresses the utilization of the endogenous reserves. In fact added lactic acid may, under certain conditions, depress the endogenous respiration (Table 4).

It may therefore be assumed that pyruvate also is utilized in preference to endogenous substrates (see Krebs & Eggleston, 1940). This would justify the calculation of the $O_2/\text{net pyruvate}$ ratios observed in the presence of fluoride on the basis of the total oxygen consumption. In the absence of fluoride, however, those ratios are often high and the endogenous respiration can be only partly suppressed by the substrate respiration.

Effect of fluoride on sperm metabolism. According to Lardy & Phillips (1941a, b) phospholipins are the endogenous substrates of bull spermatozoa. Fluoride has been shown to be an inhibitor of the oxidation of fatty acids in the liver (Jowett & Quastel, 1935), and it is possible that it also inhibits the oxidation of phospholipins. However, part of the endogenous respiration is resistant to this inhibitor. The fact that fluoride also decreases the rate of oxidative disappearance of pyruvate suggests that it may suppress a pathway of pyruvate metabolism. Since the addition of either DNP or fluoride alone depresses $Z_{\text{net pyruvate}}$ to approximately the same extent and since the effect of the two inhibitors is not additive (Table 3), it is possible that the same pathway of pyruvate metabolism is affected. Thus, by the use of fluoride, experimental conditions are established in which approximately equal amounts of pyruvate are metabolized in the presence and absence of DNP.

Effect of DNP on sperm metabolism. From the observation that DNP increased the respiration of spermatozoa metabolizing glucose, lactate or pyruvate, Lardy, Hansen & Phillips (1945) concluded 'that the preliminary dehydrogenations as well as the subsequent decarboxylative dehydrogenations are accelerated by DNP'. Analytical data reported in the present communication show that although DNP stimulates the rate of disappearance of fructose and of lactate, it does not accelerate, but in the absence of fluoride strongly inhibits, the rate of oxidative disappearance of pyruvate. In sperm suspensions with a high endogenous respiration, the oxygen consumption on addition of pyruvate represents the sum of the incompletely suppressed endogenous respiration and the respiration due to the oxidation of the pyruvate. In such samples the effect of DNP appears to be the resultant of its inhibitory effects on the endogenous respiration (see Table 1), its inhibitory effect on part of the oxidative metabolism of pyruvate (see Table 3), and its stimulating effect on the respiration associated with the residual part of the oxidative metabolism of pyruvate. As shown above, the inhibitory effects of DNP are also produced by fluoride; in the presence of fluoride the system is therefore considerably simplified, with the result that the rate of respiration can now be compared with the rate of substrate oxidation both in the absence and presence of DNP. Since, under these conditions, $Z_{\text{net pyruvate}}$ is not increased by DNP, an increased oxygen consumption must be attributed to the more complete oxidation in the presence of DNP of that fraction of pyruvate which is not reduced to lactic acid. The incomplete oxidation of the substrate has been taken to indicate its partial utilization in synthetic reactions in micro-organisms (see Clifton, 1952) and in mammary gland tissue (Turner,

1951). Similar conclusions may be drawn regarding the metabolism of spermatozoa.

Lactic acid formation and glycolysis. It has been shown in the present paper that lactic acid can be formed by spermatozoa under aerobic and anaerobic conditions not only as a result of carbohydrate breakdown, but also from pyruvate in the absence of carbohydrate. In the latter case, lactic acid is formed by a reaction which appears in the main to involve a dismutation between two molecules of pyruvate. It has been found that on addition of pyruvate to sperm suspensions incubated with fructose in the absence or presence of fluoride the rate of breakdown of carbohydrate was not accelerated, while the rate of lactic acid accumulation was increased, apparently at the expense of the added pyruvate. Since the formation of lactate by the dismutation of pyruvate is accompanied by the evolution of an equivalent amount of carbon dioxide, conclusions regarding the effect of pyruvate on the metabolism of carbohydrate in spermatozoa cannot be based on either manometric or chemical estimation of lactic acid. It has been reported by other workers that the addition of pyruvate restores the glycolysis of spermatozoa poisoned with fluoride (Lardy & Phillips, 1943b). Table 6 shows that this does not necessarily follow.

Pyruvate metabolism and semen quality. Although other factors outside the scope of this paper play a large part in determining fertility, it seems to be generally recognized that the metabolic vigour of the spermatozoa is an important property in relation to their capacity for survival and fertilization (see Mann, 1949). The experimental system described provides conditions in which certain metabolic properties of spermatozoa can be tested. DNP, which is known to inhibit synthetic reactions in bacteria (Clifton, 1946) and to abolish oxidative phosphorylation (Loomis & Lipmann, 1948), is believed to cause increased oxygen consumption owing to the inhibition of the utilization of pyruvate in synthetic reactions resulting in the more complete combustion of the substrate (see Turner, 1951). It may be expected that the viability of spermatozoa which are capable of carrying out synthetic reactions *in vitro* will be greater than that of spermatozoa whose metabolism is mainly catabolic. Measurements of respiration and substrate utilization show that samples of spermatozoa do, in fact, differ in oxidizing pyruvate either with low O_2 /pyruvate ratios, consistent with incomplete oxidation of the substrate, or with high O_2 /pyruvate ratios, indicating the complete oxidation of the substrate. Some data have been presented suggesting that there is a correlation between the metabolic behaviour of semen *in vitro* and the breeding history of the donor.

It should be pointed out that since the observed

metabolic rates represent the mean of the metabolic activities of a large number of spermatozoa, samples classified as poor may yet contain a small proportion of spermatozoa of good quality and under favourable conditions effect fertilization. In fact none of the bulls tested was completely sterile, but those of an average fertility of less than 55% are considered of little value as donors of semen for artificial insemination. Our experience suggests that the test may prove its usefulness in helping to detect bulls which produce semen of exceptionally high quality (type A), and also of semen of poor fertilizing capacity (types B and D), which because of its good appearance and motility, might otherwise be considered satisfactory. The test may also be used for periodic check on the quality of semen produced by bulls of fluctuating fertility. It may, however, not be expected to apply in cases where infertility is due to pathological or genetic causes.

Although our work had to be confined to the relatively small number of bulls available to us, the results appear to be promising enough to merit further investigation. Since it may not be possible for us to submit the test to trials on a larger scale in the near future, our results are reported at the present stage in order to make them available to other workers.

SUMMARY

1. The metabolism of pyruvate in bull spermatozoa has been studied.
2. The endogenous respiration of washed spermatozoa of good motility from different bulls varied from $-Z_{O_2}$ 6–28. Fluoride (0.02M) and 10^{-4} M-2,4-dinitrophenol (DNP) inhibited the endogenous respiration, especially when this was high.
3. In samples with low endogenous respiration, 2,4-dinitrophenol (DNP) in the presence of pyruvate increased the oxygen consumption despite some inhibition of pyruvate utilization. In samples with high endogenous respiration, DNP did not stimulate the respiration in the presence of pyruvate and strongly inhibited the oxidative removal of pyruvate. Fluoride inhibited the endogenous respiration and the oxidative removal of pyruvate to approximately the same extent as DNP. The effects of the two inhibitors were not additive.
4. In the presence of fluoride and pyruvate, DNP stimulated the respiration of certain samples in which, without fluoride, it was inhibitory. Chemical analysis showed that these samples oxidized pyruvate with low O_2 /net pyruvate ratios (<1) (net pyruvate = pyruvate utilized – lactate formed) and that DNP increased these ratios to 2.0–2.5. Other samples metabolized pyruvate with higher O_2 /net pyruvate ratios, which in some cases approached

2.5, and DNP caused a smaller or no further increase of respiration.

5. When sixty sperm samples of good motility from sixteen bulls were tested in the presence of fluoride, it was found that they could be grouped according to the type of their respiratory response to pyruvate and pyruvate + DNP. A correlation appeared to exist between the metabolic behaviour of sperm samples under the experimental conditions

described and the fertility of their donors as computed from their breeding record in artificial insemination.

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An Effect of Bicarbonate on the Oxidation of Pyruvate by Kidney Homogenates

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Mitochondrial preparations from mammalian kidney, as Green, Loomis & Auerbach (1948) have shown, are capable of oxidizing pyruvate to completion and this reaction can be coupled with the phosphorylation of adenosinemonophosphate (AMP) or adenosinediphosphate (ADP). Experiments reported in this paper show that the oxidation of pyruvate stops under some conditions at the level of acetate. The factors that control complete and partial oxidation were investigated in detail because an experimental separation of the various steps of

pyruvate oxidation is necessary in studies of the quantitative aspects of oxidative phosphorylation, a problem to be dealt with in a subsequent paper.

EXPERIMENTAL

Collection of material. In the first experiments abattoir material was collected under ice and water. Later it was found that placing the tissue in a mixture of liquid and frozen 0.9% (w/v) aqueous KCl prevented the oedema which occurred in ice and water. Under 3 min. elapsed between the killing of the animal and immersing the kidney in the