A and for the condensation and reduction of these two to yield β -hydroxybutyric acid.

2. These enzymes, which are located in the non-particulate fraction of the cell, were partially purified by ammonium sulphate fractionation.

3. The coupling of the fatty acid-synthesizing system to the transhydrogenase stimulated by oestrogen was demonstrated by the increased rate of conversion of [^{14}C]acetate into labelled fatty acid and by the increased rate of oxidation of reduced pyridine nucleotide when oestradiol was added to the system.

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The Yellow Pigmentation of Bull Semen and its Content of Riboflavin, Niacin, Thiamine and Related Compounds

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Semen is usually white or slightly creamy in colour, but during the course of unrelated studies on the physiology of spermatozoa (White, 1954) the semen from one particular Friesian bull was found to be quite yellow. The colour was confined to the plasma fraction and u.v. light produced a green fluorescence similar to that emitted by riboflavin. By visual examination, the colour of the specimen was comparable with that of an aqueous solution containing at least 10 μg . of riboflavin/ml.

Much lower values for the riboflavin content of bull semen were quoted by VanDemark & Salisbury (1944), who were presumably dealing with unpigmented specimens. The reports of Brochart (1952), Kaemmerer & Krampitz (1955) and the analyses of Mann (1954) suggest that the yellow pigmentation of bull semen may be attributed largely to riboflavin.

Recently it has been possible to obtain several samples of very yellow bull semen, and these have

provided material for more extensive investigations into the nature of the pigmentation and its effect on spermatozoa (see White & Lincoln, 1958).

EXPERIMENTAL

Semen and blood. Semen was collected from 13 bulls by the use of an artificial vagina; the semen from five was yellow and the remainder white. Frequently, two ejaculates were obtained from each animal within a few minutes. The samples were protected from light during and after collection, and precautions were taken to prevent any contact with cork, plastic or rubber. The semen was either cooled slowly to 10° or frozen to -79° before transport to the laboratory. Seminal plasma was separated from the spermatozoa by centrifuging. Spermatozoa counts, made with a haemocytometer under high power, were done in duplicate on semen samples diluted with 3% (w/v) NaCl in blood-cell pipettes.

Blood was collected from the jugular vein and the serum that separated from the clot after standing in the dark was used.

Fructose, oxygen uptake and lactic acid. Fructose and the rate of fructolysis in 0.125M-phosphate buffer, pH 7.4, prepared by dissolving 3.5 g. of Na_2HPO_4 in 190 ml. of water and adding 10 ml. of N-HCl , were estimated by the method of Mann (1948), 20 min. at 80–85° being allowed for colour development (White, 1959).

Oxygen uptake was measured manometrically by the direct Warburg technique, calcium-free Krebs-Ringer phosphate diluent being used (0.132M- NaCl , 5 mM- KCl , mM- KH_2PO_4 , mM- $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 10 mM- Na_2HPO_4). Spermatozoa were washed twice by diluting about 4 ml. of semen to 10 ml. in a tapering 15 ml. centrifuge tube and centrifuging twice at 1700 rev./min. (about 350 g) for 15 min.; the supernatant was removed after each centrifuging and replaced by an equal volume of diluent.

Lactic acid was estimated by the method of Barker & Summerson (1941).

Chromatography and electrophoresis. For chromatography or electrophoresis 1 ml. of seminal plasma was deproteinized by adding 6 vol. of ethanol and centrifuging. The solvent was removed at room temperature in a vacuum desiccator, the residue dissolved in 0.4 ml. of water and the solution clarified by centrifuging in a haematocrit tube. The solution (10 μl .) was applied to Whatman no. 1 chromatography paper along with 10 μl . of the following marker solutions (0.1 μg ./ μl .); riboflavin, riboflavin phosphate, flavinadenine dinucleotide, lumichrome and lumiflavin. The following solvents were used for the development of the chromatograms: 5% (w/v) Na_2HPO_4 (Carter, 1950); butan-1-ol-acetic acid-water (4:1:5), top phase (Cramer, 1948); phenol-butan-1-ol-water (160:30:100, w/v/v), lower phase (Dimant, Sanadi & Huennekens, 1952); methanol (Yanagita & Foster, 1956). The first three solvents were used in descending systems and the methanol in an ascending one; after drying, the spots were examined under a u.v. lamp. The electrophoresis procedure of Cerletti & Siliprandi (1955) was used.

Absorption spectrum. For the determination of the absorption spectrum of the yellow pigment, 4 ml. of seminal plasma was deproteinized with 30 ml. of ethanol. After evaporation, the residue was dissolved in 0.3 ml. of water and streaked on Whatman no. 1 chromatography paper. The chromatogram was developed with butan-1-ol-acetic acid-water and the yellow strip eluted with water by the technique of Dent, Stephen & Steward (1947). The absorption spectrum was determined in a Uvispek spectrophotometer and compared with that of riboflavin (5 μg ./ml.).

Estimation of vitamin B. The quantity of seminal plasma taken for analyses varied from 0.05 to 1.00 ml. depending upon the content of the B vitamins; 5 ml. of blood serum was used.

Thiamine, riboflavin and niacin were estimated by the procedures described in the publication, *Determination of Vitamin B₁, B₂ and Niacin in Foodstuffs* (F. Hoffman-La-Roche and Co. Ltd., Vitamin Department, Basle, 1955).

Thiamine was adsorbed onto Decalso and eluted with acidified potassium chloride before conversion into thiochrome with alkaline ferricyanide (Association of Vitamin Chemists, 1951). Thiamine loss on the surfaces of the laboratory glassware was minimized by observing the precautions described by Farrer (1953).

For the estimation of riboflavin and chloroform-soluble flavin, samples of plasma were acidified with 0.2N- H_2SO_4 , treated with 3% (w/v) KMnO_4 and decolorized with 3% (w/v) H_2O_2 . After extraction with CHCl_3 , the riboflavin in the aqueous layer was adsorbed onto kieselguhr, eluted with 2% (w/v) NaOH and converted into lumiflavin by irradiation with u.v. light from a Phillips 160 w bulb, type 57270 E/21. The riboflavin content of the sample was then estimated fluorometrically by comparison with a known amount of pure riboflavin treated similarly. A portion of the CHCl_3 layer was transferred to a cuvette for the estimation of the chloroform-soluble fluorescent material.

The Lumetron (model 402 EF) fluorimeter was used and 1 μg . of pure riboflavin or thiamine provided a suitable comparison standard. The instrument was modified by the appropriate attachments for the colorimetric determination of niacin. Niacin was estimated by the reaction with cyanogen bromide using 50 μg . of pure niacin as a standard.

Statistical analysis. Where necessary the statistical significance of the results have been assessed by the *t* test or analysis of variance (*F* test). In the latter case, sums of squares attributable to differences between ejaculates and treatments have been isolated and the interaction mean square used as the error term.

RESULTS

Preliminary observations

The production of yellow semen seems characteristic of the bulls studied, since many samples collected over several years have been pigmented. The animals were located in widely scattered parts of Australia, New Zealand and the U.S.A., and the semen of other bulls on these farms was quite normal in appearance. All bulls producing yellow semen were of the Friesian breed and nine Australian and New Zealand bulls were related, as shown in Fig. 1.

Fig. 1. Bulls producing yellow semen. The colour of the semen is shown in parentheses.

Table 1. R_f values of bull-semen pigments and markers

	Solvents			
	Butanol-acetic acid-water	5% Na_2HPO_4	Methanol	Phenol-butanol-water
Seminal plasma extract:				
Yellow spot	0.26	0.27	0.16	0.78
Pale-blue spot	0.13, 0.37, 0.65	0.06	—	0.14
Violet spot	0.86	0.00	—	0.78
Riboflavin (yellow)	0.25	0.30	0.16	0.80
Riboflavin phosphate (yellow)	0.04	0.56	—	0.15
Lumichrome (pale blue)	0.65	0.05	0.28	—
Flavinadenine dinucleotide (yellow)	0.03	0.41	0.00	0.17
Lumiflavin (yellow)	0.41	0.15	—	—

Another Australian bull of a different line (Riverdale King Romeo), producing yellow semen, is known to have two ancestors (Colony Ormsby Sir Romeo 781424 and Colony Vale Sir Romeo Fourth 832252) giving yellow semen, in the U.S.A., and an offspring of the latter (U.W. Grebegg Sir Wayne) also gives yellow semen.

The pigmentation of the semen does not affect the quality of the spermatozoa since all bulls were in use at artificial-insemination centres and were of high fertility. The morphology, fructolysis index and concentration of the spermatozoa were all normal, thus the mean ($\pm$ S.D.) rate of the fructose breakdown for nine yellow ejaculates was 1.7 ± 0.8 mg./ 10^9 sperm/hr. and the mean ($\pm$ S.D.) spermatozoa count of 13 ejaculates was

$$956 \pm 34 \times 10^6/\text{ml.}$$

The pH of the samples ranged from 6.5 to 7.0.

The yellow colour was associated with the seminal plasma rather than the spermatozoa and qualitative tests showed that the pigment had the following properties, which are characteristic of riboflavin (Sebrell & Harris, 1954): (1) a greenish fluorescence when exposed to u.v. light; (2) insoluble in chloroform, ether or benzene; (3) adsorbed on to kieselguhr from acid solutions and eluted with 2% (w/v) NaOH; (4) the colour and fluorescence were destroyed by 3% (w/v) KMnO_4 in 2% (w/v) NaOH but not by treatment with 3% (w/v) KMnO_4 for 1 min. in acid solution; (5) $\text{Na}_2\text{S}_2\text{O}_5$ destroyed the colour and fluorescence; (6) conc. H_2SO_4 produced a red colour which became yellow on dilution.

On chromatographing, the yellow pigment behaved in a similar manner to riboflavin markers in each of the four solvent systems used (Table 1). Light-blue spots, one corresponding to lumichrome, were also present and also an unknown violet spot which ran well ahead of riboflavin in butan-1-ol-acetic acid-water but remained at the origin in 5% (w/v) Na_2HPO_4 .

The absorption spectrum of the yellow pigment is shown in Fig. 2; peaks occurred at 450, 375, 255 and 210 $\text{m}\mu$.

Fig. 2. Absorption spectrum of the yellow pigment in bull seminal plasma.

Quantitative estimations

The riboflavin content of 16 yellow ejaculates from five Friesian bulls was compared with that of nine white ejaculates from nine bulls of other breeds. In the estimation of riboflavin, the preliminary chloroform extract of the yellow semen also fluoresced. This was measured quantitatively and is referred to as chloroform-soluble flavin. It is expressed as the amount of riboflavin which, on irradiation in alkaline solutions, gave an equivalent fluorescence. Thiamine, niacin and fructose analyses were also undertaken to ascertain whether these seminal constituents also differed between the two groups. The results of the vitamin analyses are set out in Table 2. The riboflavin and chloroform-soluble flavin content of the yellow samples were each, on the average, about 40 times that of the colourless semen and sufficient to account for the pigmentation. Both first and second yellow ejaculates obtained at any one time from individual bulls had a high flavin content and the values

Table 2. *Comparison of some constituents of yellow and white bull seminal plasma*

Mean values ($\mu\text{g./ml.}$) and the population standard deviation are given with the numbers of observations in parentheses.

	Riboflavin	Chloroform-soluble flavin	Niacin	Thiamine
Yellow semen	18.9 $\pm$ 6.0 (15)	19.1 $\pm$ 6.3 (15)	3.2 $\pm$ 1.7 (5)	2.8 $\pm$ 1.1 (13)
White semen	0.5 $\pm$ 0.2 (8)	0.5 $\pm$ 0.6 (8)	1.0 $\pm$ 0.2 (4)	1.2 $\pm$ 0.8 (9)

Table 3. *Effect of light on the riboflavin and chloroform-soluble flavin content of bull semen*

Values are expressed as $\mu\text{g./ml.}$ before and after incubation of the semen for 3 hr. In Expt. 2 an addition of 20 $\mu\text{g.}$ of riboflavin/ml. of semen was made. C-S flavin, chloroform-soluble flavin.

Expt. no.	Zero time		3 hr. in dark		3 hr. in light	
	Riboflavin	C-S flavin	Riboflavin	C-S flavin	Riboflavin	C-S flavin
1	21.0	19.2	21.9	19.2	0.0	11.2
1	15.5	10.4	12.9	13.2	0.3	12.0
2	40.5	17.0	41.5	18.4	9.7	7.5
2	41.0	16.0	45.5	15.6	7.5	7.5

showed little variation in individual bulls although the samples were collected over a period of several months. The ratio of riboflavin to chloroform-soluble flavin remained fairly constant at unity. Neither the niacin nor thiamine values differed significantly between the groups (Table 2) and the mean fructose value for 14 yellow ejaculates ($458 \pm 86 \text{ mg./100 ml.}$) was quite normal.

Nature of chloroform-soluble fluorescent material

The exact nature of the chloroform-soluble fluorescent material is at present unknown. Although the chloroform extracts of semen fluoresced strongly under u.v. light in a similar manner to lumiflavin, attempts to demonstrate this substance, by chromatography and electrophoresis, in ethanol and chloroform extracts of semen were not convincing. Baddiley, Buchanan & Carss (1957) have suggested that anhydriboflavin might occur in tissues but this substance, prepared by their method, could be readily separated from riboflavin by chromatography and is therefore unlikely to be the flavin in question.

The unidentified chromatogram spots (i.e. spots other than the one corresponding to riboflavin) were extracted with hot 0.2N-sulphuric acid and tested in the fluorimeter. Negative readings were obtained for all these spots, so they do not correspond with the unknown chloroform-soluble material.

Stability of semen pigment

When the semen was left on the bench for several hours, the colour faded and experiments were undertaken to ascertain whether this was due to the metabolic activity of the spermatozoa or the action of light. Samples of two freshly collected samples were exposed to direct sunlight at room temperature for 3 hr. and control samples were

Table 4. *Effect of sorbitol and ribitol on the oxygen uptake of bull spermatozoa*

Each manometer flask contained 1 ml. of washed spermatozoa suspension ($3.0\text{--}6.0 \times 10^8$ cells) in calcium-free Krebs-Ringer phosphate, incubated in air at 37° for 3 hr. The final concentration of each substrate was 1 mg./ml.

Ejaculate no.	Uptake of O_2 ($\mu\text{l./10}^8$ spermatozoa)		
	Control	Sorbitol	Ribitol
1	13.4	21.9	14.0
2	27.4	42.3	26.3
3	13.6	24.8	16.5
4	12.7	18.7	13.3
5	19.0	24.6	18.1
Mean	17.2	26.5	17.6

kept in the dark under similar conditions (Expt. 1, Table 3). The initial and final flavin content was determined. The experiment was repeated with 20 $\mu\text{g.}$ of riboflavin added to each millilitre of semen to check if an equilibrium existed between it and the chloroform-soluble flavin (Expt. 2, Table 3).

There was no breakdown of riboflavin or chloroform-soluble flavin in the dark and no evidence for an equilibrium between the two flavins in the semen. On the other hand, in the presence of sunlight much of the endogenous and added riboflavin was destroyed (F 33.4; $P < 0.01$) and the concentration of chloroform-soluble flavin also decreased (F 9.7; $P < 0.05$). Chromatography of the yellow semen before and after exposure to sunlight revealed a considerable increase in the size of the lumichrome spots.

The photolysis of riboflavin might be expected to yield ribitol. Tests with four bull ejaculates showed that the addition of 1 mg. of ribitol to 1 ml. of washed cells failed to increase the oxygen uptake

Table 5. *Effect of riboflavin on the metabolism and motility of bull spermatozoa*

Mean values and the population standard deviations are given with the numbers of observations in parentheses. Each vessel contained 1 ml. of spermatozoa suspension ($0.9-6.0 \times 10^8$ cells) in calcium-free Krebs-Ringer phosphate, incubated in air at 37° for 2-3 hr. Motility was scored by microscopic examination by the system of Emmens (1947).

	Uptake of O_2 (μ l./ 10^8 spermatozoa)	Fructose breakdown (μ g./ 10^8 spermatozoa)	Lactic acid production (μ g./ 10^8 spermatozoa)	Motility score
Control	19.7 ± 9.8 (19)	132.1 ± 89 (19)	103 ± 43 (19)	24.2 ± 10.6 (15)
Riboflavin (20 mg./ml.)	10.3 ± 6.1 (19)	104.0 ± 92 (19)	92 ± 53 (19)	17.0 ± 10.8 (15)

(Table 4) under conditions where sorbitol was effective (F 36.5; $P < 0.01$). It seems unlikely therefore that bull spermatozoa are able to oxidize ribitol.

Concentrations of blood riboflavin

Determinations were made of the concentration of riboflavin and of chloroform-soluble fluorescent material in the blood of three bulls which produced yellow semen. The average values were 0.1 and 0.6 μ g./ml. respectively.

Effect of riboflavin on the metabolism and motility of spermatozoa

When one part of normal white bull semen was mixed with 1-4 parts of calcium-free Krebs-Ringer phosphate containing fructose at a concentration of 100 mg./100 ml. and sufficient riboflavin to give a final concentration of 20 μ g./ml. the oxygen uptake of the suspension was significantly decreased (Table 5; t 3.6; $P < 0.01$).

The mean values for fructose breakdown, lactic acid production and motility are also less in the presence of riboflavin but the response was variable and the differences between control and riboflavin-treated samples are not statistically significant.

DISCUSSION

The results clearly establish that the yellow coloration of some bull ejaculates is due to high concentrations of riboflavin. The pigmentation of the semen is not due to dietary factors, as other bulls under the same conditions of husbandry produce normal semen and the concentrations of blood riboflavin were not elevated. On the other hand, the facts that all the bulls producing yellow semen were of the same breed and many were related, strongly suggest that the characteristic is hereditary and that it may behave like a Mendelian dominant. In view of the blood analyses, the tissues generally of these bulls are probably not capable of synthesizing the flavins. It seems more likely that we may be dealing with a mutation in which the accessory organs, probably the seminal vesicles,

have the specific ability to make or concentrate both riboflavin and chloroform-soluble flavin.

Chloroform-soluble lumiflavin is known to be formed from riboflavin when irradiated under alkaline conditions. The chloroform-soluble flavin found in the seminal plasma could not, however, be produced by the photolysis of riboflavin after ejaculation since similar amounts of the two flavins were present even when the most rigorous precautions were taken to exclude sunlight from the semen during collection and handling. Further, bull semen is slightly acid, which would tend to favour photolysis to lumichrome, a process which accompanies the fading of these ejaculates. The enzymic formation of chloroform-soluble flavin from riboflavin after ejaculation is improbable but the accessory organs may contain an enzyme system capable of interconverting riboflavin and the chloroform-soluble material.

The mechanism of the repression of the oxygen uptake of bull spermatozoa by riboflavin is a subject for future investigation. Preliminary tests have indicated that the effect may be largely abolished by the addition of catalase to the diluent. It is possible therefore that the riboflavin may be reduced by the spermatozoa or some seminal constituent and reoxidized on shaking in the Warburg flask with the production of traces of hydrogen peroxide. The oxygen uptake of bull spermatozoa is known to be particularly sensitive to hydrogen peroxide (Tosic & Walton, 1950; Wales, White & Lamond, 1959).

The inability of intact bull spermatozoa to utilize ribitol and at the same time to utilize sorbitol (White, 1957) is surprising since sorbitol-dehydrogenase preparations from bull spermatozoa apparently have at least some activity with ribitol as substrate (King & Mann, 1958).

SUMMARY

1. The seminal plasma from a number of otherwise normal Friesian bulls was quite yellow and the evidence suggests the characteristic is hereditary and may behave like a Mendelian dominant.

2. The yellow pigment has been identified as riboflavin which occurred at an average concentration of 19 $\mu\text{g./ml.}$ in pigmented samples compared with 0.5 $\mu\text{g./ml.}$ in colourless samples. Similar amounts of chloroform-soluble fluorescent material were also present.

3. The niacin, thiamine and fructose levels of the yellow samples were not significantly elevated.

4. Exposure to light caused the yellow semen to fade and this was accompanied by the breakdown of riboflavin to lumichrome. Ribitol, which might be expected to form in the process, is not oxidized by bull spermatozoa.

5. When riboflavin was added to normal white ejaculates at a concentration of 20 $\mu\text{g./ml.}$ it decreased the oxygen uptake and decreased the motility of the spermatozoa.

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Metabolism of Amines

2. THE OXIDATION OF NATURAL POLYAMINES BY *MYCOBACTERIUM SMEGMATIS**

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The degradation of natural polyamines by various bacteria has been described by Razin, Gery & Bachrach (1959). *Pseudomonas aeruginosa* was found to oxidize spermine and spermidine, γ -aminobutyric acid and β -alanine being intermediates in the reaction. Roulet & Zeller (1945) had already reported that *Mycobacterium smegmatis* was capable of oxidizing spermine. It was therefore decided to study polyamine oxidation by this

organism, and to compare the oxidation pathway with that of *P. aeruginosa* (Razin *et al.* 1959).

The degradations of γ -aminobutyric acid and β -alanine, which are intermediates in the oxidation of the polyamines, are also described in this paper.

MATERIALS AND METHODS

Organisms. *Mycobacterium smegmatis* strain 10143 was obtained from the American Type Culture Collection. *Pseudomonas aeruginosa* was the strain used previously (Razin *et al.* 1959).

* Part 1: Razin, Gery & Bachrach (1959).