functions thus releasing them for a more specific purpose in relation to carbohydrate metabolism. This might result in more efficient utilization of glucose and so in the recovery of consciousness at a lower level of blood glucose.

SUMMARY

1. Oral administration of L-glutamic acid to patients in hypoglycaemic coma was without effect except in one subject.

2. Intravenous injection of 20 g. of L-glutamic acid restored consciousness to subjects in hypoglycaemic coma in 26 of 45 experiments, and modified the depth of coma in the remaining 19 experiments.

3. Similar effects were produced by the injection of 20 g. of aminoacetic and p-aminobenzoic acids.

4. In all cases where amino-acids were injected intravenously into hypoglycaemic subjects there was a rise in blood glucose, which was, however, in itself inadequate for the restoration of consciousness.

5. Injection of L-glutamic acid into subjects in hypoglycaemic coma was without significant effect on blood urea.

6. Injection of succinic acid into hypoglycaemic subjects was without significant effect on blood glucose or on state of consciousness.

7. Intravenous injection of L-glutamic acid into non-hypoglycaemic subjects failed to produce any significant effect on blood glucose, but produced vomiting far more strongly than in hypoglycaemic subjects.

We wish to express our gratitude to Dr P. K. McCowan, Physician Superintendent of Crichton Royal, for his interest in our work and for his permission to publish the results. Our thanks are also due to Miss A. Beattie, sister-in-charge of the Insulin Department, for her collaboration.

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Studies on the Metabolism of Semen

5. CITRIC ACID IN SEMEN

By G. F. HUMPHREY AND T. MANN, *Molteno Institute, University of Cambridge*

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The semen of man and certain other higher mammals is distinguished by a very high content of citric acid (Scherstén, 1929, 1936; Dickens, 1941; Huggins & Neal, 1942; Lardy & Phillips, 1945; Barron & Huggins, 1946*a*, *b*; Humphrey & Mann, 1948). The acid originates in the accessory glands of reproduction, chiefly the seminal vesicles, and in this respect it resembles another more recently discovered component of semen, namely fructose, which has similarly been shown to be secreted mainly in the seminal vesicles (Mann, 1946). The present study was undertaken primarily with the object of investigating the possibility that there may exist a link between the two substances with regard to their formation, distribution or function in the reproductive organs and semen. In the course of this study it was established that the process of generation and maintenance in semen of both fructose and

citric acid is closely dependent upon and regulated by the same hormone, testosterone. At the same time it was found that, in certain species at least, fructose and citric acid originate in different parts of the reproductive system and that their levels in semen may vary independently of each other. Moreover, in distinction to fructose, citric acid has been found to be metabolized in semen much more slowly than fructose. These and other facts concerning the relation of citric acid to both the anaerobic and aerobic metabolism of semen will be described and discussed in this paper (preliminary communication, Humphrey & Mann, 1948).

METHODS

The material consisted of semen and reproductive organs from several species including ram, bull, boar, stallion, cock, rat and rabbit. The separation of semen into seminal plasma and spermatozoa, and the preparation of washed sperm suspensions were carried out as previously described (Mann, 1945, 1946). Ringer-bicarbonate used in experiments to determine the respiratory quotients was made by adding 0·154M-$NaHCO_3$ to the Ringer solution, to give a final bicarbonate concentration of 0·008M, so that when in equilibrium with 95% O_2 and 5% CO_2 the pH of the medium was 7·0. Trichloroacetic acid was employed as deproteinizing reagent for both semen and accessory reproductive organs.

Determinations of O_2 uptake were made in Barcroft differential manometers and in Warburg manometers at 37°. The respiratory quotients were measured in Warburg manometers by the indirect method (Dixon, 1943). Anaerobic experiments were conducted in Thunberg tubes filled with pure N_2 or with 95% N_2 and 5% CO_2, and the manometric estimation of acid production was carried out in Barcroft differential manometers with gas outlets by measuring the CO_2 output using Ringer-bicarbonate and a gas mixture of 95% N_2 and 5% CO_2. Fructose and fructolysis were determined as described previously (Mann, 1946, 1948). Citric acid was estimated by the method of Pucher, Sherman & Vickery (1936) as modified by Krebs & Eggleston (1944), and the specificity of the method checked according to Breusch & Tulers (1947). Good agreement was obtained between the titrimetric and colorimetric procedures, but the latter was used as a routine. Succinic acid was analyzed according to Krebs, Smyth & Evans (1940), and lactic acid by the method of Friedemann, Cotonio & Shaffer (1929).

RESULTS

Content of citric acid in semen and reproductive organs

Citric acid constitutes a major component of the whole ejaculated semen in several mammalian species including bull, ram, boar, stallion and rabbit. However, with the exception of rabbit, it is usually absent from the epididymal semen, and only small quantities of it are found in ampullar semen. A particularly high concentration of citric acid in whole semen is characteristic for bull where it may exceed 1%, and also for rabbit and ram. All these three species also show relatively high seminal fructose contents. However, the semen of boar and stallion, which shows relatively low fructose contents, contain much citric acid (130 and 55 mg./100 ml. respectively). On the other hand, dog and cock semen appear to be almost entirely devoid of both fructose and citric acid. The species most completely examined was the ram; altogether 48 samples of semen were analyzed during the seven months' breeding season extending from October to May. The lowest value was 66 mg. citric acid/100 ml. semen, the highest 261 mg./100 ml. The monthly average was higher in October at the beginning of the season (196 mg./100 ml.) than late in April when the season was coming to a close (92 mg./100 ml.). If two collections of semen were made in quick succession from the same ram, the result was as shown in Table 1. It can be seen that unlike fructose, which was usually higher in the second ejaculate than in the first, citric acid did not always show the same regularity.

Table 1. *Fructose and citric acid in successive ejaculates of ram semen*

Date of collection	Ram (no.)	Ejaculate (no.)	Fructose (mg./100 ml.)	Citric acid (mg./100 ml.)
10 Oct.	1	1	586	261
		2	800	226
	2	1	484	107
		2	674	178
	3*	1	474	83
		2	536	66
12 Dec.	1	1	262	168
		2	364	192
	4	1	328	192
		2	364	144

* This ram had been irradiated with artificial light throughout the previous winter by Dr Yeates and used by him for the study of the effect of light on the reproductive cycle in sheep (Yeates, 1947).

Although both fructose and citric acid are generated in the same part of the reproductive system, the accessory glands, they can be shown to be secreted by functionally and anatomically distinct tissues. The citric acid contents of the various reproductive organs of full-grown animals are given in Table 2. It can be seen that, in rabbit for instance, citric acid is met with principally in the glandula vesicularis rather than in the prostate organ. Yet as previously shown, fructose reaches a higher concentration in the rabbit prostate than in the glandula vesicularis (Davies & Mann, 1947). An even clearer picture was obtained through the study of the accessory glands in the rat which revealed a high concentration of citric acid in the seminal vesicle as well as in the ventral prostate. Yet both these organs are poor in fructose

(9 and 0 mg./100 g.). In the rat, fructose is concentrated mainly in two other organs, namely, the dorsolateral prostate (82 mg./100 g.) and the small gland adjacent to the seminal vesicle proper, known as the 'coagulating gland' or 'anterior prostate'. The coagulating gland, thus called because of the presence in it of the semen-coagulating enzyme 'vesiculase', is distinguished by a complete absence of citric acid; at the same time it had a high fructose content (172 mg./100 g.).

Table 2. *Distribution of citric acid in male reproductive organs*

Species	Material	Citric acid (mg./100 g. tissue)
Boar:	Secretion from Cowper's gland	0
	Prostate	38
	Epididymal semen	0
	Secretion from seminal vesicle	580
Bull:	Testis	3
	Epididymis	18
	Secretion from the seminal gland	670
	Ampullar semen	550
	Epididymal semen	0
Rabbit:	Epididymis	54
	Testis	15
	Glandula vesicularis	84
	Secretion of glandula vesicularis	834
	Prostate (I, II and III)	62
	Cowper's gland	42
	Ampulla	273
Rat:	Seminal vesicle proper	39
	Coagulating gland	0
	Ampulla	0
	Dorsolateral prostate	20
	Ventral prostate	122

Table 3. *Effect of castration and testosterone treatment on citric acid content of rabbit organs*

	Citric acid in combined tissues of glandula vesicularis, prostate and ampullae (mg./100 g.)
Non-castrated buck	105
One week after castration	73
Two weeks after castration	22
Five weeks after castration and simultaneous implantation of testosterone propionate (100 mg.)	108
Castrated and implanted simultaneously with testosterone; five weeks later pellet removed; rabbit killed after another five weeks	20

Effect of testicular hormone on the formation of citric acid

The level of citric acid in semen and in male reproductive organs is dependent foremost on the degree of sexual maturity of the animal. It is low in young animals which have not yet reached maturity, and is generally linked with the extent of activity of the male sex hormone in the animal body. On castration there is a gradual decline in the citric acid content of accessory glands, and, unless testosterone is applied by injection or implantation, the organs soon become almost depleted of citric acid.

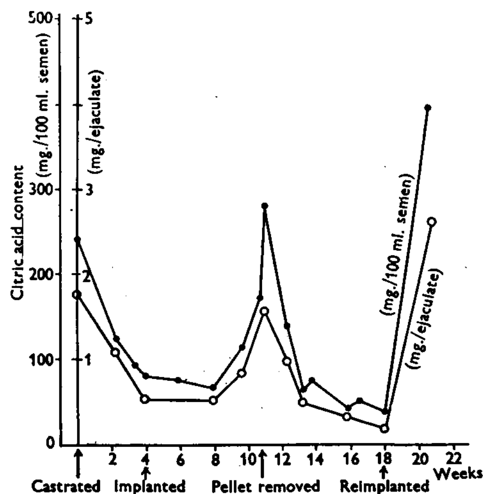

Fig. 1. Effect of castration and implantation of testosterone on the citric acid content of rabbit semen. Buck castrated when 18 months old; 4 weeks later implanted subcutaneously with a pellet of testosterone propionate (100 mg.); 7 weeks later pellet removed, dried, weighed (80 mg.) and preserved; 7 weeks later same pellet reimplanted into the same animal.

This can be seen from data recorded in Table 3, which were obtained by analysis of the accessory glands in a series of five full-grown male rabbits. A similar, even clearer, picture of the correlation between citric acid level and testosterone activity was obtained by a direct analysis of the seminal fluid collected by means of the artificial vagina. By this means it was possible to follow closely the sequence of changes brought about by castration and hormone implantation in the intact animal. Fig. 1 illustrates the effect of castration and of subsequent implantation of testosterone on the level of citric acid in the semen of the same rabbit; one curve shows the changes in the concentration of citric acid in semen (mg./100 ml.) and the other the changes in the absolute quantity of citric acid as represented by a whole single ejaculate. Both curves show clearly the post-castration fall of citric acid in semen and the

recovery which followed implantation of testosterone. A point which deserves special attention is that, as a result of the prolonged treatment with testosterone, the formation of citric acid in the castrated buck has been raised to a level beyond that usually observed in a non-castrated and untreated animal.

Citricolysis

Citricolysis, by which we mean the metabolic breakdown and disappearance of citric acid, was studied (*a*) in whole semen, by incubating it *in vitro* and allowing it to use up the preformed citric acid; and (*b*) in washed sperm suspensions incubated with added citric acid. Unlike fructolysis, which proceeds in spermatozoa at a constant and characteristic rate and which can be conveniently expressed in terms of a well-defined 'fructolysis index' (Mann, 1948), the rate of utilization of citric acid was not constant enough to warrant the introduction of a definite 'index'. In any case, in whole semen the rate of citric acid disappearance was found to be much smaller than that of fructose. In bull and ram semen, for instance, the rate of citric acid disappearance was frequently as low as 0·05 mg./hr. at 37° in the presence of 10^9 sperm cells, as against 1·5–2·0 mg. fructose used up under identical conditions. Occasionally, the rate of citricolysis was higher. This is shown by the experiments recorded in Table 4 in which whole ram semen was used (4×10^9 sperm, and 1·3 mg. citric acid/ml.), as well as washed spermatozoa of the same concentration; the decrease in citric acid resulting from incubation for 2 hr. at 37° is expressed in Table 4 as a percentage of that initially present. It may be added here that, although in whole semen as ejaculated, citric acid appears to be distributed in both the cells and the plasma, the spermatozoa can be easily freed from citric acid by washing with Ringer solution. For instance, the suspension of washed spermatozoa as used in the experiment referred to above was prepared by diluting ram semen with 3 vol. of Ringer solution, centrifuging and washing the cells with 2 vol. of Ringer. The washed cells were found to be free from citric acid.

Table 4. *Citricolysis in ram spermatozoa*

	Percentage decrease in citric acid content as result of 2 hr. incubation at 37°		
	In air	In O_2	In N_2
Whole semen	70	73	60
Whole semen diluted with 2 vol. Ringer-phosphate	40	31	35
Washed sperm suspension (4×10^9 cells/ml.) with 0·1% citric acid and 0·2% fructose added	30	34	33

The process of citricolysis, although rather sluggish, may continue in semen for some time after the spermatozoa have exhausted the entire reserve of seminal fructose, i.e. after fructolysis has come to an end. This was noted both in aerobically and anaerobically incubated semen. Active citricolysis was found to be linked with the presence of sperm cells in semen, the seminal plasma itself being unable to metabolize citric acid. Indeed seminal plasma contains a heat-labile factor which generally inhibited the oxidation of citrate by animal tissues. This inhibitory effect was particularly noticeable when the seminal plasma was added to a liver pulp which alone utilized citrate very efficiently. This is illustrated by the following experiment. Rat liver was ground with 9 vol. 0·1 M-phosphate buffer, pH 7·4, and three 2 ml. samples of tissue pulp were shaken aerobically for 90 min. at 37°, with the following additions: (i) Ringer solution (0·8 ml.), (ii) Ringer solution (0·8 ml.) containing citrate (5 mg. citric acid), and (iii) seminal plasma (0·8 ml. with a content of 5 mg. citric acid). The results (Table 5) show that the seminal plasma had a pronounced inhibitory effect on both O_2 uptake and citrate utilization by the ground liver tissue; dialyzed, but not heated, seminal plasma, caused a similar inhibition.

Table 5. *Effect of bull seminal plasma on the O_2 uptake and citrate utilization by liver pulp*

	Results of 90 min. incubation	
Additions to liver pulp	O_2 uptake (μl.)	Decrease in citric acid (%)
Ringer solution	750	—
Ringer with added citrate*	990	87
Seminal plasma	120	5

* Equal to the amount present in the seminal plasma.

However, unlike the citrate oxidation in liver pulp, that in intact cells such as the spermatozoa was not interfered with by the seminal plasma, and proceeded at approximately the same slow rate in sperm suspensions as in whole semen.

An attempt was made to identify succinic acid as a possible intermediary product of the aerobic metabolism of citric acid in spermatozoa. Samples (2 ml.) of washed sperm suspensions (2×10^9 ram sperm/ml. Ringer-phosphate) were incubated aerobically for 3 hr. in Barcroft manometers, in presence and absence of citrate, with and without the addition of 0·005 M-malonate. No accumulation of succinic acid was observed in any of these samples (Table 6). It may also be added that the level of succinic acid in fresh bull and ram seminal plasma was determined; it was found to be rather low (5–10 mg./100 ml. plasma).

Table 6. *Effect of malonate on aerobic metabolism of citrate by ram spermatozoa*

Additions to the sperm suspension	Changes resulting from 3 hr. incubation: O_2 uptake (µl.)	Citric acid disappearance (mg.)	Succinic acid formation (mg.)
None	1040	—	—
Citric acid (2·8 mg.)	1045	0·41	0
Citric acid (2·8 mg.) plus malonate (0·005 M)	1190	0·57	0
Malonate (0·005 M)	1170	—	0

Citric acid in relation to respiration and fructolysis in semen

Under conditions so far studied, no appreciable effect of citric acid on either fructolysis or respiration of washed sperm suspensions could be detected. This is borne out by the experiment (cf. Table 7) which was carried out with a suspension of washed ram spermatozoa in Ringer-phosphate, $0{\cdot}7 \times 10^9$ cells/ml.

The suspension was divided into two parts of which one (*A*) was treated with 480 mg. fructose/100 ml., and the other (*B*) with 100 mg. fructose/100 ml. Each of the two suspensions was then divided into two parts, one of which was left without any further treatment, whilst the other received 180 mg. citric acid (neutralized)/100 ml. All four samples were incubated at 37° in narrow test tubes (0·7 cm. in diameter, as used for storage of semen required for artificial insemination) and the disappearance of fructose was followed at 30 min. intervals.

No significant difference in the rate of fructose disappearance between sperm suspensions containing citrate and those incubated in absence of citrate was observed (cf. Table 7). A similar experiment was

Table 7. *Effect of citrate on fructolysis in washed ram spermatozoa*

	Fructose concentration in sperm suspension (mg./100 ml.)			
	Suspension *A**		Suspension *B*	
Incubation (min.)	Without citrate	With citrate	Without citrate	With citrate
0	480	480	100	100
30	414	415	62	61
60	361	346	27	27
90	294	304	15	12

* Suspensions *A* and *B* contained different amounts of added fructose (see text).

carried out with the four sperm suspensions in Ringer-bicarbonate instead of Ringer-phosphate and using Barcroft manometers filled with 5 % CO_2 and 95 % N_2, instead of the narrow-bore tubes employed above. This made it possible to follow fructolysis by manometric measurement of acid production in addition to the chemical estimations of fructose and lactic acid. Again, no difference could be detected in the rate of fructose disappearance between citrate-free and citrate-containing suspensions, and both produced lactic acid at the same rate. No lactic acid, however, was produced by spermatozoa from citrate alone, i.e. when addition of fructose was omitted.

Previous investigations (Mann & Lutwak-Mann, 1948) have shown that the O_2 uptake of dilute suspensions of washed spermatozoa declined gradually unless the sperm cells were provided with an extracellular source of oxidizable material such as a glycolyzable sugar or lactic acid. In the present study,

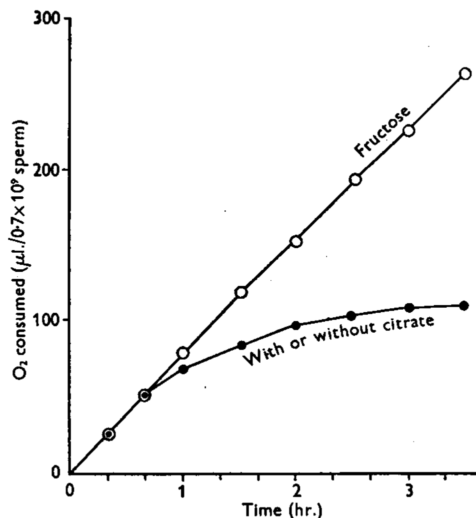

Fig. 2. Effect of fructose and citric acid on the respiration of washed ram spermatozoa.

observations were extended to a number of other compounds and in particular to organic acids. Several organic acid salts such as acetate, propionate, butyrate, oxaloacetate and pyruvate, were found to have a pronounced beneficial influence on the respiration of spermatozoa, in accordance with the view held by Lardy & Phillips (1944, 1945). However, the action of these organic acids as revealed by our study was not on the initial rate of respiration and did not result in any marked rise of the initial O_2 uptake. Instead, these substances were found to maintain and prolong the initial rate of respiration, i.e. to delay the decline in O_2 consumption which would otherwise take place. Unlike the glycolyzable sugars and the above-mentioned organic acids, citric acid was found to be unable to maintain the rate of O_2 uptake of ram spermatozoa (Fig. 2). In this respect it resembled lactose, ethanol, glycerol, glycine, succinic acid and malic acid, none of which exhibited any marked 'initial rate preserving effect' on the sperm respiration.

The possibility was also examined that citrate may have a stimulating effect on the R.Q. of spermatozoa. However, unlike fructose, the addition of citrate had, if anything, a slight depressing effect on the R.Q. of washed ram sperm.

In the course of this study the question was also taken up of possible variations in the respiratory activity of spermatozoa induced by changes in O_2- and CO_2-tension. It was found that the value of Z_{O_2} (μl. O_2 taken up by 10^8 sperm cells in 1 hr.) remained unaltered when the O_2 tension was lowered from 100 to 4%. On the other hand, the sperm respiration was higher in the presence of 5% CO_2 and 95% O_2 than in either air or pure O_2. The R.Q. and Z_{O_2} values shown in Table 8 were all obtained using washed sperm suspensions with a final concentration of $2{\cdot}5 \times 10^8$ ram spermatozoa/ml.

Table 8. R.Q. *and* Z_{O_2} *of washed suspensions of ram sperm*

Duration (hr.)	No additional substrate	In presence of 0·01 M-citrate	In presence of 0·01 M-fructose
		R.Q.	
1	0·92	0·83	1·00
2	0·78	0·75	0·91
3	0·84	0·75	0·79
Gas		Z_{O_2} at the end of 1 hr.	
In pure O_2	11·6	12·0	14·0
In 95% O_2–5% CO_2	15·6	14·0	19·2

Formation of citric acid in spermatozoa and reproductive organs

In the course of their study on fat and carbohydrate oxidation in mammalian spermatozoa, Lardy & Phillips (1945) demonstrated the presence of aconitase in bull sperm as well as the ability of sperm cells to form citric acid from added pyruvate or oxaloacetate. Using washed ram spermatozoa we were able to confirm these observations. The analysis of citric acid synthesis convinced us, however, that the quantitative contribution of spermatozoa, as regards the final content of citric acid in semen, was very slight or negligible, and that the bulk of the citric acid must be derived from the secretions of the accessory glands rather than from the spermatozoa. In our experience there was no difficulty in completely freeing bull and ram spermatozoa from citric acid by washing them once or twice with Ringer solution. We were also unable to detect any appreciable formation of citric acid on incubation of the washed spermatozoa. In order to induce the sperm cells to form citric acid, a large concentration of added oxaloacetate was found to be necessary. The formation of citric acid from oxaloacetate by washed suspensions of ram spermatozoa is illustrated in Table 9.

In this experiment 4 ml. ram semen were diluted with 16 ml. Ringer solution, centrifuged, and the sperm suspended in sufficient Ringer solution to give a final volume of 8 ml.; this reduced the concentration of preformed citric acid to 0·06 mg./ml. Part of the suspension was diluted with an equal volume of 0·1 M-Na-oxaloacetate in Ringer solution and kept for 1 hr. at 37°. Another sample of the sperm suspension was first inactivated by heating for 2 min. in boiling water and then mixed and incubated with oxaloacetate, serving as a control experiment for the non-enzymic conversion of oxaloacetate to citric acid.

From Table 9 it can be seen that under the conditions studied no citric acid was formed in the heat-inactivated sample. However, the unheated sperm cells produced citric acid from oxaloacetic acid, and the synthesis was greater under aerobic than anaerobic conditions. Aerobically, as much as 0·21 mg. citric acid was produced in 1 hr. by 10^9 ram spermatozoa. Assuming that the dry weight of 10^9 sperm is 30 mg. we arrive at the figure of 0·7 mg. citric acid produced by 100 mg. sperm, dry weight.

Table 9. *Formation of citric acid from oxaloacetic acid by washed suspensions of ram spermatozoa*

Pretreatment of sperm	Incubation in	Citric acid content (mg./ml.)	Citric acid formed (mg./ml.)	Citric acid formed (mg./10^9 sperm)
Heat inactivation	N_2	0·06	—	—
None	N_2	0·18	0·12	0·07
Heat inactivation	Air	0·06	—	—
None	Air	0·42	0·36	0·21

Table 10. *Anaerobic formation of citric acid from oxaloacetate by rat seminal vesicle and testis*

	Citric acid formed (mg./g. tissue)
Seminal vesicle	
Heat-inactivated pulp	0·00
Fresh pulp	0·12
Testis	
Heat-inactivated pulp	0·00
Fresh pulp	1·32

A similar study of the citrate synthesis from oxaloacetate was also made with two rat organs, the testis and the seminal vesicle.

The tissues were ground with 5 parts 0·1 M-phosphate buffer, pH 7·3, and the suspensions diluted with an equal volume of Ringer solution containing 6·5 mg. glucose, 75 mg. oxaloacetic acid (Na salt) and 8·5 mg. Na pyruvate/ml. The mixtures were then incubated in Thunberg tubes filled with N_2, and citric acid determined after 1 hr. at 37°. Control experiments were run simultaneously with mixtures containing heat-inactivated tissue suspensions.

The analytical results (Table 10) showed that under these conditions the rat testis was more than

ten times as active as seminal vesicle in synthesizing citric acid from the added keto acids. Similar results were obtained with aerobically incubated tissue suspensions, but less significance can be attached to the aerobic experiments in view of the slow, but nevertheless definite, disappearance of citric acid from air-incubated suspensions of ground seminal vesicles.

Regarding the content of aconitase in semen, this was studied separately in washed sperm suspensions and in seminal plasma.

Undiluted whole ram semen was centrifuged, the seminal plasma separated, and the cells washed twice with Ringer solution. The plasma and the centrifuged sperm were then separately diluted with Ringer-phosphate up to the original volume of whole semen. To 0·1 ml. samples of 'plasma' or 'sperm', 0·3 ml. Ringer-phosphate and 0·4 ml. 0·13 M-phosphate buffer, pH 7·4, containing 3 mg. *cis*-aconitic acid (Na salt) were added, and the mixtures were kept for 30 min. at 30° in Thunberg tubes filled with N_2.

The results (Table 11) showed that the seminal plasma was devoid of aconitase activity. The ram spermatozoa, on the other hand, showed themselves capable of efficiently converting *cis*-aconitic into citric acid, the citric acid formation, expressed in terms of $Q_{citrate}$ (μl. citric acid formed in 1 hr. by 1 mg. tissue dry wt.) being 10·7.

Table 11. *Aconitase activity in spermatozoa and seminal plasma*

	Incubation (min.)	Citric acid formed (mg.)	Aconitic acid converted to citric acid (%)
Sperm suspension	30	1·39	42
Seminal plasma	30	0·00	—

To enable some comparison between the aconitase activity of spermatozoa and that of other animal tissues to be made, two rat organs, liver and seminal vesicle, were assayed for their aconitase content. The tissues were thoroughly ground with 5 parts 0·1 M-phosphate buffer and centrifuged; 1 ml. extract of 1:50 diluted liver extract and 1 ml. of 1:10 seminal vesicle extract were each treated with *cis*-aconitate and incubated as described above for seminal plasma and spermatozoa. The values for $Q_{citrate}$ were 17·2 for rat liver, and 3·6 for rat seminal vesicle.

DISCUSSION

The seminal plasma, i.e. the composite mixture of secretions from the male accessory glands, serves both as a vehicle and nutrient for spermatozoa in whole ejaculated semen; chemically it differs from most other body fluids in several respects. The seminal plasma is remarkable for its high content of three chemical substances, citric acid (Scherstén, 1929), fructose (Mann, 1946) and phosphorylcholine (Lundquist, 1946), all of which appear to originate in the same accessory organ, the seminal vesicle. In this respect, however, the rule is not without exceptions, as shown by our study of rabbit and rat generative organs. In the rabbit, citric acid was met with principally in the glandula vesicularis, whereas the highest concentration of fructose was found in the prostate. In the rat, citric acid was located in the ventral prostate and the seminal vesicle proper, but fructose in the dorsal prostate and in the coagulating gland. Furthermore, it was found that a high concentration in semen of one component does not necessarily run parallel with a correspondingly high level of the other. Boar and stallion semen, for instance, although rich in citric acid, were shown to be comparatively poor in fructose. Moreover, there are daily individual fluctuations in the level of citric acid and fructose in semen, and they were shown not to coincide with each other; also a variable ratio citric acid/fructose was encountered and found to be characteristic of semen. On the whole it seems probable that citric acid of semen is secreted independently of fructose, in the sense that these two seminal components are produced by two distinct types of secretory cells. In one respect, however, the processes of citric acid and fructose generation in the male accessory organs closely resemble each other. As demonstrated in this study, both citric acid and fructose are formed under the influence of, and in close dependence upon, the male sex hormone. Thus the behaviour of citric acid is analogous to that previously described for fructose (Mann & Parsons, 1947), except that the post-castration disappearance and the hormone-induced reappearance of citric acid in seminal plasma are not as prompt as in the case of fructose.

Fructose in semen represents a source of readily available energy for the spermatozoa (Mann, 1946, 1948; Mann & Lutwak-Mann, 1948). With regard to citric acid, however, so far its function is rather obscure and the clarification of this problem must await further study. It is conceivable that citric acid may be associated with the phenomenon of spontaneous gelification, coagulation and subsequent liquefaction which normally occur in the semen of certain species. In this connexion one may recall the finding of Huggins & Neal (1942) that citrate in human semen is the cause of prolonged coagulation time of mixtures of blood and seminal fluid, and that this delay of clotting can be effectively counteracted by adding calcium ions. The possibility of citrate acting as a binding substance for calcium has been envisaged by Scherstén (1936) and Huggins (1945), and the fact pointed out that milk, another fluid rich in citrate, has also a high calcium content. In the same connexion our observations may be recalled that in the rabbit citric acid occurs mainly in the glandula vesicularis, that is, in the organ which is

associated with the process of semen gelification. Similarly, in the rat, a high concentration of citric acid was found in the seminal vesicle proper, the organ which normally provides the substrate for vesiculase leading to gel formation.

Hyaluronidase is another enzyme to be considered in connexion with the possible role of citric acid in semen. We carried out some experiments and found that hyaluronidase activity of washed ram spermatozoa was increased by citrate to the same extent as by an equimolar solution of sodium chloride. However, it is possible that the activity of this enzyme is linked with citrate in a more indirect manner, such as that indicated by Baumberger & Fried (1948), who claim that citrate exerts a 'protective action' against antinvasin *in vitro*.

There is no indication that citric acid in semen acts as a major source of nutrient material for the spermatozoa. The rate, both aerobically and anaerobically, at which citric acid is broken down by spermatozoa, whether in whole fresh semen or when added to sperm suspensions, is slight in comparison with the rate of normal fructolysis. When both fructose and citrate were supplied to washed spermatozoa, citrate failed to exert a sparing effect on fructose, and fructolysis went on at exactly the same rate in presence and absence of citrate. Citrate unlike glucose, lactate, pyruvate, oxaloacetate or acetate, was ineffective in maintaining the rate of respiration of washed spermatozoa. On the other hand, however, citricolysis, although slow, continued even after the spermatozoa had used up all available fructose and this, together with the earlier finding of Lardy & Phillips (1945) that citrate has some beneficial effect on the sperm motility, suggests that citrate may be of some specific value to sperm metabolism.

The spermatozoa of the ram contain aconitase, ($Q_{citrate} = 10{\cdot}7$), and they are able to form citric acid from added oxaloacetic acid. However, the bulk of citric acid present in whole ejaculated semen is derived from the seminal plasma and not from the spermatozoa. It is interesting to note that under the experimental conditions employed, the rat seminal vesicle, which normally contains more citric acid than, e.g., liver, testis or spermatozoa, was found to have less aconitase activity than these tissues and little ability to form citrate from oxaloacetate. In this respect, the rat seminal vesicle differs from the human prostate which is the chief citric acid producing organ in man, and which, according to Barron & Huggins (1946*a*, *b*), is particularly rich in aconitase. Before any general conclusions can be drawn concerning the mechanism of citrate production in the male organs of reproduction, it would be essential to extend the investigations to several more animal species. It is also necessary to bear in mind the possibility that accessory glands of reproduction may contain factors similar to that found in bull seminal plasma which was shown to interfere with the metabolism of citric acid in liver pulp.

SUMMARY

1. Citric acid is a major component of semen and the concentration is particularly high in bull semen where it may exceed 1 %. It is usually absent from epididymal semen but is present in ejaculated and sometimes also in ampullar semen. It is derived mainly from the secretions of the accessory glands of reproduction, chiefly from the seminal vesicle but also from the prostate.

2. There is no definite correlation between the processes of fructose and citric acid formation in the accessory organs. In the rat and rabbit the two substances are found in two different types of secretory organs.

3. Following castration seminal citric acid disappears, but it reappears in response to testosterone. However, the post-castration fall of citric acid as well as its reappearance after testosterone treatment are less prompt than in the case of fructose.

4. When fresh semen is incubated *in vitro* citric acid is metabolized by spermatozoa both aerobically and anaerobically. The rate of citrate utilization, however, is much smaller than that of fructose. Similarly, washed spermatozoa utilize added citrate much more slowly than added fructose. Seminal plasma itself is unable to metabolize citric acid. It contains a heat-labile factor which inhibits O_2 uptake and citrate oxidation in liver pulp.

5. Citric acid has no effect on the course of fructolysis by washed ram spermatozoa. It is incapable of maintaining the sperm respiration; in this respect it differs from fructose as well as from other organic acids such as lactic, pyruvic, oxaloacetic, acetic, propionic and butyric, all of which prolong the respiration of washed spermatozoa. The R.Q. of spermatozoa is little affected by citrate, but is increased to 1 by fructose.

6. Ram spermatozoa contain aconitase and are able to form citrate from added oxaloacetate. However, the bulk of citric acid present in whole ejaculated semen is derived from the seminal plasma and not from the spermatozoa. The seminal plasma is devoid of aconitase activity.

7. Rat seminal vesicles, despite their high content of citric acid, appear to have comparatively little enzymic activity associated with the formation of citrate from oxaloacetate.

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A Note on the Disturbance of the Haemoglobin Metabolism of the Rat by Sulphanilamide

By J. W. LEGGE, *Institute of Medical Research, Royal North Shore Hospital, Sydney**

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Certain disturbances of haemoglobin metabolism were among the side reactions observed after the introduction of sulphonamide therapy. Animal experiments clearly demonstrated that continued dosage produced an anaemia. In human subjects cyanosis was common, due mainly to methaemoglobinaemia and occasionally to sulphaemoglobinaemia. Drug rashes were also observed in a proportion of cases, and, while not all of the rashes were associated with light sensitivity, the excretion of urinary porphyrin after dosage was found to be increased in a number of cases (cf. Rimington & Hemmings, 1938). The excretion of porphyrin in the urine of the rat was then found to be increased many times by dosage with sulphonamides (Wien, 1938; Rimington & Hemmings, 1938, 1939). Other aromatic amino compounds were found to produce a porphyrinuria. Rimington & Hemmings (1939) observed a correlation between the severity of the porphyrinuria and the presence of methaemoglobin in the blood of their animals. Since the porphyrin was found to be coproporphyrin type III, differing only from the protoporphyrin IX found in haemoglobin in having propionic acid side chains in place of vinyl side chains, they put forward the tentative hypothesis that the coproporphyrin was derived from the increased haemoglobin breakdown under these conditions. A similar view was entertained by Brownlee (1939) as a result of his investigation of the effects following the administration of antipyretics to rats. The appearance of coproporphyrin would signify, according to this hypothesis, a departure from the normal pathway of haemoglobin catabolism.

The present work was begun in order to see if this porphyrinuric action of the sulphonamides in rats could be mitigated by the administration at the same time of substances, such as ascorbic acid, hydrochloric acid, etc., which had been claimed to diminish the severity of the side reactions following sulphonamide therapy. In addition, it was felt desirable to investigate quantitatively the bile-pigment excretion during dosage with the drug so that an estimate might be made of the importance of the normal pathway of haemoglobin catabolism under these conditions. Rimington & Hemmings (1938) had indeed commented on the large amounts of urobilin which they observed in the course of their analyses of faecal porphyrins.

* Now at the Biochemistry Department, Melbourne University.