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Studies on the Metabolism of Semen

8. ERGOTHIONEINE AS A NORMAL CONSTITUENT OF BOAR SEMINAL PLASMA. PURIFICATION AND CRYSTALLIZATION. SITE OF FORMATION AND FUNCTION

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The discovery of ergothioneine is due to Tanret (1909) who isolated it from ergot of rye and established its composition as $C_9H_{15}N_3O_2S$. Barger & Ewins (1911) identified the new substance as a betaine of thiolhistidine (β-2-thiolglyoxaline-4(5)-propiobetaine). They were also the first to point out that the sulphur of thiolglyoxaline is readily oxidized by ferric chloride or bromine to free sulphuric acid; this property, which distinguishes ergothioneine from cysteine and glutathione, has been utilized for the determination of ergothioneine (Blumenthal & Clarke, 1935; Touster, 1951). The final confirmation of the structure of ergothioneine was provided by Heath, Lawson & Rimington (1950, 1951) who succeeded in synthesizing ergothioneine from 2-thiolhistidine, an amino-acid which had been obtained synthetically (Ashley & Harington, 1930; Harington & Overhoff, 1933) but has hitherto never been found in nature and which is unable to replace histidine as a growth-promoting factor in animals (Neuberger & Webster, 1946).

Ergot, from which ergothioneine can be obtained in yields varying from 65 to 260 mg./100 g. (Eagles, 1928; Pirie, 1933*a*; Hunter, Molnar & Wight, 1949), remained the only known source of ergothioneine until Hunter & Eagles (1925, 1927) isolated from pig blood the crystalline substance X, later renamed sympectothion which, although different from uric acid, gave with phosphotungstic and arsenophosphotungstic acid reagents the same blue colour reaction as uric acid. A blood constituent with similar properties, named thiasine, was isolated independently by Benedict, Newton & Behre (1926). Both sympectothion and thiasine were soon shown to be identical with ergothioneine (Newton, Benedict & Dakin, 1926; Eagles & Johnson, 1927). Blood ergothioneine, or 'thioneine' as it is sometimes called, is confined entirely to the erythrocytes and does not occur in the plasma. A sensitive and specific method for its estimation based on the diazo reaction has been developed by Hunter (1928, 1949). The content of ergothioneine in blood (mg./100 ml.) is much smaller than in ergot; it varies from 1·8 to 1·95 in man, 1·3 to 3·1 in rat, and 2·0 to 26·5 in pig (Hunter, 1951).

The present study reports the discovery of a third source of ergothioneine in nature, namely the seminal plasma (preliminary communication: Leone & Mann, 1951). Protein-free extracts from semen have been known for a long time to exhibit a marked reducing property towards 2:6-dichlorophenolindophenol, but this fact has usually been attributed to ascorbic acid (cf. Berg, Huggins & Hodges, 1941). We were able to confirm the occurrence of ascorbic acid in semen, but in addition we detected the presence of yet another reducing substance which has hitherto escaped recognition by other workers. This substance is ergothioneine. It occurs in a particularly high concentration in the semen of boar, and it originates in the seminal vesicles, the glands which also secrete fructose (Mann, 1946), citric acid (Humphrey & Mann, 1949) and inositol (Mann, 1951). In the secretion of the seminal vesicles, ergothioneine is present in a dialysable form. The

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concentration of ergothioneine in the boar vesicular secretion is much higher than in the blood of any species including pig, and in some instances was found to approach the level in ergot. Thus, boar vesicular secretion represents a convenient starting material for the preparation of crystalline ergothioneine. It is important to stress that, unlike blood, the semen carries ergothioneine as an extracellular constituent.

MATERIAL AND METHODS

Boar semen and vesicular secretion. A single boar semen ejaculate varies from 150 to 500 ml., but has only a low density of spermatozoa (25000–300000/μl.) and its large bulk is made up by the seminal plasma. According to McKenzie, Miller & Bauguess (1938), the relative contributions to the semen volume are: 15–25% by the seminal vesicles, 10–20% by Cowper's glands, 55–70% by the prostate and urethral glands and only 2–5% by sperm and epididymal fluid. In the present study, whole semen, as ejaculated, was collected by means of an artificial vagina. Epididymal semen, i.e. the thick sperm suspension stored in the cauda epididymidis, was obtained from the glands by incision; the sperm density of the epididymal semen was 2000000–5000000/μl. The vesicular secretion was removed, either by incision or by means of a syringe, from the vesicles of freshly slaughtered animals; the seminal vesicles, the weight of which varied from 100 to 920 g., contained between 70 and 560 ml. secretion.

Ram and bull semen were collected by means of an artificial vagina (Walton, 1945). The semen was used whole or it was separated by centrifugation into seminal plasma and spermatozoa. The preparation of washed sperm suspensions was carried out as previously described (Mann, 1946).

Quantitative determination of ergothioneine. The method used as a routine for the quantitative determination of ergothioneine in whole semen, seminal plasma and in the male accessory gland secretions, was that described by Hunter (1949), and the diazo reaction was carried out following closely Hunter's (1951) procedure in extracts prepared by deproteinization with either $Zn(OH)_2$ or 66–80% ethanol. Occasionally parallel estimations were made, some by the method of Brown (1945) which depends on the Folin-Marenzi reaction of ergothioneine with the 'uric acid reagent', and others by the method of Touster (1951) in which the thiol group is oxidized with Br_2 to sulphate, which is next precipitated as benzidine sulphate and the benzidine content of the precipitate determined colorimetrically with sodium 1:2-naphthaquinone-4-sulphonate, according to Letonoff & Reinhold (1936).

Chromatographic detection of ergothioneine, and differentiation from thiolhistidine, uric acid and ascorbic acid

Paper partition chromatography has proved a useful means for the detection of small amounts of ergothioneine (5 μg. or even less). Chromatograms were run at room temperature for 24 hr. on Whatman no. 1 filter paper, with butanol-acetic acid as solvent (40 vol. *n*-butanol + 10 vol. acetic acid + 50 vol. water; upper layer used). The spots were located on the dry paper chromatograms by photographic detection of ultraviolet-absorbing areas, following a procedure which is similar to that described by Markham & Smith (1949) for the chromatographic detection of purines; the source of ultraviolet light was a mercury arc lamp with a high emission at 254 and 265 mμ., and the photographic paper used was Kodak reflex document paper. When the photographic images were obtained on the contact print, the areas on the paper chromatogram corresponding to the spots on the photographic print were marked. The paper chromatogram thus obtained was sprayed with one of the reagents which give colour reactions with ergothioneine, thiolhistidine, uric acid or ascorbic acid. The following reagents were used.

Diazo reagent (Pauly, 1904; Koessler & Hanke, 1919). The reagent was prepared by mixing immediately before use 2 parts of a solution containing 9 g. sulphanilic acid and 90 ml. conc. HCl/l. with 1 part of 5% (w/v) $NaNO_3$ and 1 part of 20% (w/v) NaOH. The spot produced by diazotized sulphanilic acid with ergothioneine is bright red, with thiolhistidine light orange and with histidine bright yellow with a red margin.

Phosphotungstic acid reagent (Folin & Marenzi, 1929; Work, 1949). The paper chromatogram was sprayed first with a solution prepared by mixing equal volumes of 50% (w/v) urea and 12% (w/v) NaCN, and a few minutes later with a phosphotungstic acid solution prepared according to Brown (1945). Both ergothioneine and thiolhistidine produced a blue coloration with the phosphotungstic acid reagent in a manner similar to uric acid from which, however, they could be distinguished by the position of the spots.

2:6-*Dichloroquinone-chloroimide* (Fearon, 1944; McAllister, 1951). Ergothioneine was identified and distinguished from uric acid by spraying the paper chromatogram first with 0·125 N-Na_2CO_3 and then with a 0·4% (w/v) ethanolic solution of 2:6-dichloroquinone-chloroimide. The colour produced was crimson red with ergothioneine; uric acid gave a yellow-orange spot.

Ammoniacal silver nitrate spray (Trevelyan, Procter & Harrison, 1950). The reagent as used for the detection of sugars reacted also with ergothioneine, to give a characteristic pale spot in contrast to the much darker spots due to various sugars and inositol.

2:6-*Dichlorophenolindophenol* (Mapson & Partridge, 1949). This reagent when applied to paper chromatograms makes it possible to distinguish ergothioneine from other reducing substances, in particular, ascorbic acid. In a similar category was the ferric sulphate reagent used by Strohecker & Sierp (1950) for ascorbic acid determination; this reagent was applied by spraying the paper first with 10% (w/v) KCNS and a few minutes later with a solution which was prepared by dissolving 0·38 g. ferric sulphate in water, adding 2 ml. 20% (v/v) H_2SO_4, and diluting with water to 400 ml.

Ninhydrin, known to react with thiolhistidine (Dent, 1948), gave a completely negative reaction with ergothioneine.

Other analytical methods

Fructose and fructolysis in semen were assayed as described before (Mann, 1948). Lactic acid was determined by the method of Friedemann, Cotonio & Shaffer (1929). The O_2 uptake of spermatozoa was determined in Barcroft manometers. Thiocyanate was determined according to Goldstein (1950), ascorbic acid by the method of Roe & Kuether (1943).

RESULTS

Purification and crystallization of ergothioneine from the boar vesicular secretion

The vesicular secretion as collected directly from the boar seminal vesicle is a milky, highly viscous fluid, the specific gravity of which is usually between 1·04 and 1·06. The dry weight content is of the order of 15–20 %, of which as much as one-fourth or even one-third is made up of dialysable material. Out of the several methods tested, the two outlined below were finally chosen for the isolation of ergothioneine from the vesicular secretion as yielding the most satisfactory results.

Method 1. The vesicular secretion is diluted with 2 vol. of water, and the bulky mucous precipitate removed 15 min. later by centrifugation. The centrifuged supernatant is deproteinized with $Zn(OH)_2$ by treating it with 1/10 vol. of 5% (w/v) $ZnSO_4.7H_2O$ and neutralizing with N-NaOH dropwise, with constant stirring. The deproteinized mixture is then immersed in boiling water and the stirring continued long enough to maintain the temperature at 93–97° for at least 2 min., before filtering through coarse filter paper. The filtrate, which should be water-clear or only very slightly opalescent, is made 0·5 N by the addition of the required quantity of 10 N-H_2SO_4, the precipitate (if any) removed by centrifugation, the supernatant solution cooled to about 10° and treated with 50% solution of phosphotungstic acid (w/v) in 0·5 N-H_2SO_4 until the precipitation is complete. After standing overnight in the refrigerator, the precipitate, which contains ergothioneine, is separated by centrifugation, washed with cold 0·5% solution of phosphotungstic acid in 0·5 N-H_2SO_4, resuspended in a small amount of water, and ground in a mortar with powdered $Ba(OH)_2$ until the mixture becomes permanently alkaline (blue to thymol blue). It is then centrifuged, the excess of Ba^{2+} removed by addition of H_2SO_4 until the reaction becomes slightly acid (orange to methyl red), spun again, and to the supernatant solution 4 vol. of ethanol added. After standing overnight at room temperature, the ethanol-precipitable material is removed by centrifugation, and the clear, slightly yellowish solution is concentrated to a syrup in a crystallizing dish over phosphorus pentoxide. On gradual addition of ethanol and cooling, crystallization sets in. The first crystalline fraction is obtained by allowing the crystallization to proceed in the presence of 50% ethanol for 24 hr. at 0°. After this fraction has been collected, the ethanol concentration in the filtrate is increased to 75% when a second crop of crystals comes down. The final, third fraction is obtained in presence of 90% ethanol. Each fraction is washed with ethanol and dried, and analysed separately for ergothioneine content. The second crystalline fraction showed the highest absolute content and concentration of ergothioneine.

With the use of the above described procedure, and starting in each case from 1300 ml. of the boar vesicular secretion, we have carried out the isolation of ergothioneine on two separate occasions, and obtained each time about 0·7 g. crystalline material 80% pure.

Method 2. The boar vesicular secretion is treated without any preliminary extraction, with 3 vol. of ethanol, centrifuged, and filtered if necessary. The ethanolic extract is concentrated *in vacuo* at 45° to one-tenth of the original volume, centrifuged, and precipitated with 4 vol. of ethanol. Hardly any ergothioneine is precipitated under these conditions. The ethanolic extract is concentrated *in vacuo* until ethanol is removed and in the aqueous solution ergothioneine is precipitated with phosphotungstic acid in the presence of 0·5 N-H_2SO_4. From here onwards, the steps of purification are essentially the same as in method 1; the yield and purity of ergothioneine fractions obtained in this manner are similar to those stated before.

Final purification and identification of ergothioneine. The final purification of seminal ergothioneine depended entirely on repeated recrystallization from 66–70% ethanol. 0·48 g. pure ergothioneine was obtained from 0·7 g. of the 80% pure preparation. The composition of the purest preparation was C, 47·1; H, 6·6; N, 18·2; S, 14·0. Calc. for $C_9H_{15}N_3O_2S$: C, 47·1; H, 6·6; N, 18·3; S, 14·0%. The analysis of total sulphur (carried out by Drs Weiler and Strauss) and the analysis of bromine-oxidizable sulphur by the method of Touster (1951) gave identical results; inorganic sulphate was altogether absent. The nitroprusside test and the ninhydrin test were negative. The phosphorus content was nil. Chromatography revealed no trace of thiolhistidine or of any other ultraviolet-absorbing material. On treatment with potassium hydroxide, carried out according to Barger & Ewins (1911), trimethylamine was obtained. Treatment with mercuric chloride, following the method of Tanret (1909), gave the crystalline Hg derivative of ergothioneine. All principal reactions of crystalline seminal ergothioneine including the diazo, phosphotungstic and dichlorophenolindophenol reactions were compared with similar reactions given by pure synthetic ergothioneine and pure ergothioneine obtained from ergot. No difference was found in their behaviour.

Content of ergothioneine in reproductive organs, semen and other tissues and body fluids

Content of ergothioneine in the boar vesicular secretion. The quantitative determination of ergothioneine in vesicular secretion was carried out on samples from twenty boars. The contents ranged from 29 to 256 mg./100 ml., but out of twenty values, fifteen were within the range of 50–100, and the average figure calculated from all results was 79 mg./100 ml. In most cases, the value obtained by Hunter's diazo method was only a little less than the figure calculated on the basis of Brown's phosphotungstic acid reduction method. Moreover, ergothioneine, as determined by the diazo method, accounted for the major portion of the entire reducing power as assessed by titration with dichlorophenolindophenol. The difference between the total reduction value and that due to ergothioneine was due mostly to ascorbic acid. This can

be seen from the following example in which the total reducing value is expressed as 100 %, and the other results given as percentage of that value.

Total reduction (determined by titration with dichlorophenolindophenol in a trichloroacetic acid-metaphosphoric acid extract from the boar vesicular secretion) ≡ 100 %. Reduction due to ergothioneine (calculated from the result of ergothioneine determination by the diazo method in the zinc filtrate) ≡ 85 %. Reduction due to ascorbic acid (calculated from the estimation of ascorbic acid by the method of Roe & Kuether, 1943) ≡ 12 %. Reduction due to phosphotungstic acid-reducing substances including ergothioneine (calculated from results obtained by the method of Brown (1945)) ≡ 88 %.

Absence of thiolhistidine in the boar vesicular secretion. In view of the high concentration of ergothioneine in the boar vesicular secretion, and the close chemical relationship between ergothioneine and thiolhistidine, a search was made for thiolhistidine in the vesicular secretion as well as in seminal vesicle tissue itself. To solve the problem, paper chromatography was applied in conjunction with the photographic detection of ultraviolet-absorbing spots. Both thiolhistidine and ergothioneine absorb ultraviolet light, but since their R_F values differ it is possible to resolve mixtures of them completely. However, no thiolhistidine was detected in either the protein-free extracts from the vesicular secretion or in the protein residue, directly or after acid hydrolysis. Similarly, no thiolhistidine was found in acid hydrolysates from the glandular tissue of the seminal vesicles.

Content of ergothioneine in other accessory gland secretions and in boar semen. In contrast to the vesicular secretion, other accessory gland secretions of the boar were found to contain a negligible amount of ergothioneine; boar epididymal semen (representing sperm and the epididymal secretion) 0·2–2 mg./100 ml.; prostatic secretion and Cowper's gland secretion 0·2–1·5 mg./100 ml. Whole boar semen as ejaculated contained on the average about 20 mg. ergothioneine/100 ml. but there were considerable variations between ejaculates even in the same individual. Sperm separated from the boar semen by centrifugation carried only a small content of ergothioneine; the bulk was confined to the seminal plasma.

Urinary ergothioneine. The occurrence of ergothioneine in human urine has been a matter of controversy for some time (Sullivan & Hess, 1933; Work, 1949; Woolf, 1949; Lawson, Morley & Woolf, 1950). An analysis was carried out with concentrates of both human and boar urine prepared according to Work (1949), by means of the chromatographic and detection procedures previously described. The presence of ergothioneine in urine should result in a definite ultraviolet-absorbing area on the paper chromatogram, capable of reacting with the diazo reagent, the phosphotungstic acid reagent and other colour-yielding reagents. Using this procedure, we were able to locate on the chromatograms from urine several spots which were either ultraviolet-absorbing or which reacted with one or the other of the spraying reagents; however, the behaviour of the ultraviolet-absorbing areas in relation to the colour-yielding spots was such as to indicate the occurrence of no more than traces of ergothioneine. This was particularly so in the case of human urine which contained less than 1 mg. ergothioneine/100 ml. On one occasion, boar urine showed some ergothioneine, certainly no more than 2 mg./100 ml., but at all other times less than 1 mg./100 ml. was found.

Blood ergothioneine. The ergothioneine content of boar blood was determined in three animals. The results were 6·1, 3·1 and 4·2 mg./100 ml., respectively.

Ergothioneine in foetal fluids and certain other tissues. The foetal fluids share with the seminal plasma the unusual property of containing fructose as a normal major constituent. However, this chemical resemblance did not extend to ergothioneine. Pig foetal fluids were analysed on two occasions, in a 30-day-old, and in an 80-day-old foetus. There was no ergothioneine in either of these samples. A negative result was also obtained from the analysis of ergothioneine in the pig corpora lutea and adrenal glands, two organs notorious for their high level of ascorbic acid. No ergothioneine could be detected in the pig thyroid gland or in the vitreous fluid of the eye.

The semen of bull, ram and man. Chromatographic analysis of concentrates from bull semen and bull seminal vesicle secretion revealed only a very weak ultraviolet-absorbing and diazo-staining area which could be ascribed to ergothioneine, although at the same time it showed considerable reducing power as assessed by the dichlorophenolindophenol titration and by colour reaction with the phosphotungstic acid 'uric acid reagent'. Ascorbic acid, as estimated by the Roe & Kuether (1943) procedure, accounted for 90 % of the dichlorophenolindophenol reducing substances, but did not account for more than 25 % of the value obtained by the determination with phosphotungstic acid. For the time being, the nature of the phosphotungstic acid-reactive material in bull semen remains obscure. However, a recent investigation by Leone (1952) showed that on treatment of bull-semen concentrates with purified uricase, the colour reaction with phosphotungstic acid markedly decreased. This indicates that some, at least, of the phosphotungstic acid-reactive material is uric acid. Ergothioneine could not be found, except perhaps in traces, in the specimens of

ram and human semen so far examined. On the other hand, in both these species chromatographic analysis revealed the presence in semen of some other diazo-reactive compounds. The relationship between these compounds and ergothioneine remains to be investigated.

Ergothioneine in relation to metabolism and motility of spermatozoa

There is good reason to believe that sulphydryl groups are involved in sperm activity (MacLeod, 1941, 1946; Barron, Nelson & Ardao, 1949), and in the case of human spermatozoa, cysteine and

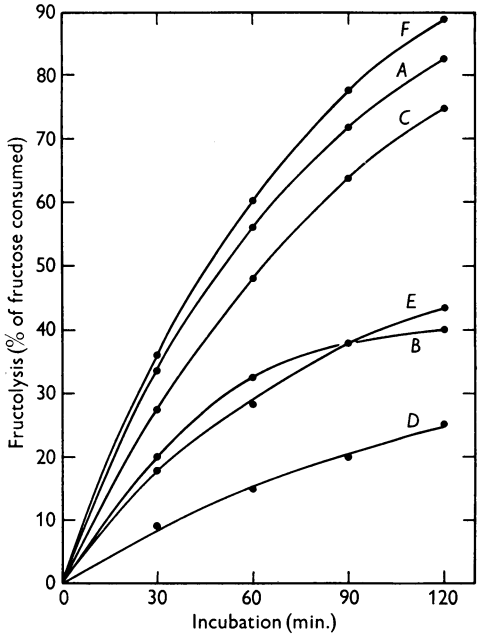

Fig. 1. Anaerobic fructolysis at 37° in ram semen diluted 12 times with Ringer phosphate to which fructose has been added (0·7 mg./ml.). *A*, no additions; *B*, $CuSO_4$ (2×10^{-4} M); *C*, $CuSO_4$ (2×10^{-4} M) + ergothioneine (3×10^{-3} M), added immediately after $CuSO_4$; *D*, H_2O_2 (10^{-3} M); *E*, H_2O_2 (10^{-3} M) + ergothioneine (3×10^{-3} M), added immediately after H_2O_2; *F*, ergothioneine (3×10^{-3} M).

glutathione have both been shown to protect *in vitro* the sperm motility and glycolysis from the inhibitory action of cupric ions (MacLeod, 1951). So far as the boar is concerned, cysteine and glutathione have not been found in the vesicular secretion in any significant quantities. In view, however, of the high content of ergothioneine in the boar vesicular secretion and seminal plasma, it was thought possible that this substance may be capable of protecting spermatozoa against the paralysing action of SH-binding inhibitors.

Experiments were first carried out with ram spermatozoa. The anaerobic fructolysis in dilute ram semen (Fig. 1) was strongly inhibited by 2×10^{-4} M-copper sulphate and by 10^{-3} M-hydrogen peroxide. However, by the simultaneous addition of 3×10^{-3} M-ergothioneine, it was possible to prevent the inhibition, particularly with Cu^{2+}. It will also be noticed that ergothioneine by itself, that is in absence of inhibitors, had a distinctly beneficial influence on the anaerobic rate of fructose utilization. This type of experiment was repeated with the inclusion of a series of semen samples incubated aerobically. Table 1 shows that aerobic fructolysis

Table 1. *Aerobic and anaerobic fructolysis in ram semen*

(Semen diluted 12 times with Ringer phosphate containing 0·7 mg. fructose/ml., and incubated at 37° for 120 min. in air and in N_2 respectively. Fructose content was determined at the end of the incubation period.)

	Decrease in fructose (%)	
Sperm with	Aerobic	Anaerobic
No additions	45	73
Ergothioneine (3×10^{-3} M)	46	76
$CuSO_4$ (2×10^{-4} M)	28	34
$CuSO_4$ + ergothioneine	34	71
H_2O_2 (10^{-3} M)	0	0
H_2O_2 + ergothioneine	5	63

responded to the action of the inhibitors and ergothioneine respectively, in a manner analogous to that demonstrated for the anaerobic process. In this particular experiment, ergothioneine was very effective in counteracting the inhibition caused by hydrogen peroxide. As a rule, however, satisfactory and reproducible results were obtained not so much with hydrogen peroxide, as with Cu^{2+} or with other sulphydryl reagents such as, for instance, *o*-iodosobenzoic acid; 10^{-3} M-sodium iodosobenzoate completely inhibited fructolysis in ram spermatozoa, but the addition to the reaction mixture of 2×10^{-3} M-ergothioneine just before or immediately following the inhibitor, prevented the inhibition. When the addition of ergothioneine was postponed until after the addition of iodosobenzoate, fructolysis could not be fully restored.

Having established the mutually antagonistic action between ergothioneine and the SH group-affecting inhibitors in ram sperm, we carried out a series of experiments with boar sperm. Boar spermatozoa separated from whole ejaculated semen by centrifugation, washed to remove the seminal plasma, and suspended in Ringer phosphate-fructose, utilized fructose at a higher rate in the presence than in the absence of added ergothioneine. Like the fructolysis of ram sperm, that of the boar sperm was sensitive to SH-inhibitors in the absence, but not in the presence, of suitable con-

centrations of ergothioneine. As already mentioned, there is no ergothioneine in the boar epididymis; thus, under physiological conditions, the spermatozoa come in contact with ergothioneine only after having left the epididymis, that is in the course of ejaculation, when they mix with the vesicular secretion. This circumstance offers an excellent opportunity for a comparative study of ejaculated versus epididymal sperm, under the influence of SH-binding inhibitors and ergothioneine,

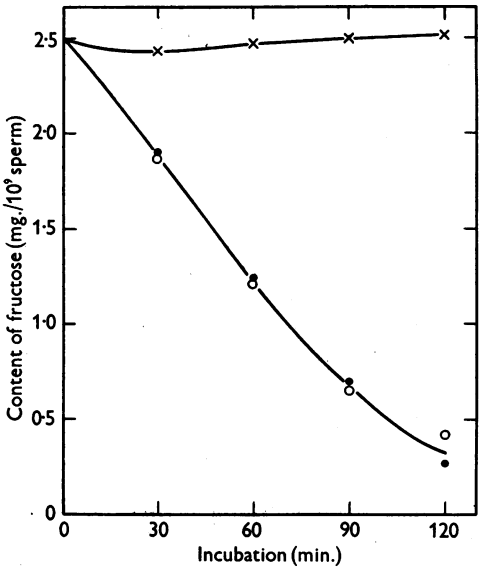

Fig. 2. Anaerobic fructolysis at 37° in boar epididymal spermatozoa to which fructose was added, 2·5 mg. fructose/10^9 sperm; ○—○, no additions; ×—×, iodosobenzoate (10^{-3} M); ●—●, iodosobenzoate (10^{-3} M) + ergothioneine (2×10^{-3} M).

respectively. Fig. 2 illustrates the result of an experiment carried out with boar sperm taken directly from the epididymis and diluted with Ringer phosphate-fructose. The utilization of fructose by the epididymal spermatozoa was brought to a standstill by 10^{-3} M-iodosobenzoate, but the inhibition could be entirely prevented by the addition of 2×10^{-3} M-ergothioneine, corresponding to 46 mg./100 ml., a concentration of the same order of magnitude as found *in vivo* in the boar vesicular secretion.

Fructolysis measurements in spermatozoa were always carried out in conjunction with observations on motility. It was found that Cu^{2+} and iodosobenzoate exerted the same paralysing effect upon motility as upon anaerobic and aerobic fructolysis; spermatozoa could be protected from loss of motility by the same concentration of ergothioneine as that required for the preservation of fructolysis. This relationship, however, was rather more complicated in the case of another SH-binding inhibitor, namely *p*-chloromercuribenzoate which appeared to affect motility before its influence on the progress of fructolysis became evident.

Some experiments were carried out on similar lines with ergothioneine as an antagonist of SH-inhibitors, in tissues other than semen. It was found, for instance, that lactic acid formation in a Meyerhof extract from rabbit skeletal muscles, which was strongly inhibited by SH-inhibitors, proceeded unchanged in the presence of added ergothioneine. Similarly, SH-inhibitors were unable to exert their inhibitory effect on the alcoholic fermentation in baker's yeast when ergothioneine was present.

With reference to the experiments on the protective action of ergothioneine, it is of interest that the intensity of colour reactions characteristic of ergothioneine such as the diazo reaction, was markedly reduced in the presence of oxidizing thiol-reagents such as *o*-iodosobenzoic acid, thus indicating a direct chemical interaction between ergothioneine and these substances.

DISCUSSION

For many years, the evidence for the occurrence of ergothioneine in the animal body rested almost entirely on findings with blood erythrocytes. The discovery of ergothioneine in the seminal vesicle secretion of boar, described in the present study, establishes a second, and incidentally a much richer, source of ergothioneine in the animal body. In addition, it represents the first instance of an extracellular occurrence of this substance in a body fluid of animal origin.

Among the characteristic features of ergothioneine worth noting is the blue colour reaction with Folin's phosphotungstic acid reagent, similar to that given by uric acid, and a high reducing value towards 2:6-dichlorophenolindophenol. The normal oxidation potential of ergothioneine is 0·36 V., as against 0·27 V. for cysteine, 0·32 V. for thiol-histidine, and 0·45 V. for glutathione (Ryklan & Schmidt, 1944). Ergothioneine was shown by Pirie (1933*b*) to share with various substituted thiocarbamides and related substances, the ability to catalyse *in vitro* the oxidation of cysteine and glutathione by hydrogen peroxide.

So far as semen is concerned, it is necessary to remember that observations made on the semen of one animal species do not necessarily apply to other species. There is an analogy to this in the striking morphological differences between the spermatozoa of different species. Thus, the high reducing power of bull semen is largely due to ascorbic acid, of boar semen to ergothioneine, and of semen from ram or

man to a variety of reducing substances. Further complications arise from the variable chemical character of the seminal plasma within one species, and even in the same individual, the chief reason for such behaviour being the peculiar make-up of the seminal plasma as a mixture of secretions from several accessory glands characterized by a fluctuating output (Mann & Lutwak-Mann, 1951).

In boar semen, ergothioneine has been found to originate chiefly in the seminal vesicles, and for this reason the purification and crystallization of seminal ergothioneine was carried out with the boar vesicular secretion as starting material. The chemical identification of the isolated product rests on the results of the elementary analysis, on the preparation of several chemical derivatives, including the crystalline mercury compound, and on the demonstration of all the chief chemical properties required of pure ergothioneine, such as the characteristic content of readily oxidizable sulphur; the reducing power towards a variety of reagents including dichlorophenolindophenol and Folin's 'phosphotungstic acid reagent for uric acid'; and the striking diazo reaction which makes it possible to demonstrate the presence of ergothioneine in a few drops of the boar vesicular secretion.

Work on the biogenesis of seminal ergothioneine is now in progress, but three facts which may be relevant have already been established, namely the absence of thiolhistidine, either free or bound, in the vesicular secretion and in the seminal vesicle tissue; the absence of thiocyanate; the absence of inorganic sulphate. In fact, ergothioneine accounts for nearly all of the non-protein sulphur in the boar vesicular secretion. Future investigations will no doubt answer the question as to the endogenous or exogenous origin of ergothioneine in boar semen. Similar issues are, of course, involved in the problem of ergothioneine occurrence in the blood. Of all mammals, pig has the highest concentration of ergothioneine in the blood. On the basis of nutritional studies it has been claimed that blood ergothioneine is exogenous (Eagles & Vars, 1928; Potter & Franke, 1935); variations in blood ergothioneine according to geographical location have been observed by Hunter (1951). An increased level of ergothioneine in the blood of diabetics has been reported by Benedict *et al.* (1926), Salt (1931) and Fraser (1950).

Concerning the possible physiological role of ergothioneine in semen, it is at the moment by no means easy to give an adequate answer. However, the following points merit attention. Boar semen, with its characteristically high content of ergothioneine, differs further from that of other animals by its very large volume and, at the same time, low density of spermatozoa. These properties have a direct bearing on the short survival time and poor 'keeping quality' of boar sperm *in vitro*, and limit at present the value of boar semen in the practice of artificial insemination. Moreover, the period of time required for the completion of a single ejaculation in the boar is much longer than in other animals. In addition, boar semen as a whole, that is sperm and seminal plasma together, is ejaculated directly into the uterus. Possibly all, or some at least, of these circumstances may be involved in the relationship between the high content and the physiological role of ergothioneine in boar semen.

The results of past investigations by Brachet (1944), Barron *et al.* (1949), and MacLeod (1941, 1946) have given an indication of the importance of SH-groups in spermatozoa for their normal motility and metabolism. In his study on human sperm, MacLeod (1951) was able to demonstrate the protective effect of cysteine and glutathione against the Cu^{2+} inhibition of motility and glycolysis. However, a similar protective action of ergothioneine is of considerably greater physiological significance, in view of the actual occurrence of ergothioneine in the seminal plasma. The present study provided evidence that ergothioneine can counteract most effectively the paralysing action of several thiol-reagents including Cu^{2+} and *o*-iodosobenzoic acid, on the metabolism and motility of spermatozoa. Of particular interest is the antagonistic effect of ergothioneine towards hydrogen peroxide, in view of the demonstration by Tosic & Walton (1950) that toxic amounts of hydrogen peroxide are formed under certain conditions by the spermatozoa themselves in the course of their aerobic metabolism. So far, no adequate evidence has been furnished for a specific function of ergothioneine in blood. However, Spicer, Wooley & Kessler (1951) found that the rates of methaemoglobin formation and reduction in nitrite-treated erythrocytes were related inversely to the blood ergothioneine levels. It is conceivable that one at least of the functions of ergothioneine in both semen and blood may be the maintenance of intracellular SH-groups in a physiologically active condition.

SUMMARY

1. Ergothioneine has been found in boar semen. It is a constituent of the seminal plasma and not of the spermatozoa themselves. It is produced in the seminal vesicles, and does not occur in any other accessory gland secretions.

2. Ergothioneine in the boar vesicular secretion accounts for the major part of the total reducing material which reacts with 2:6-dichlorophenolindophenol in the cold, and which yields the blue colour with Folin's 'phosphotungstic acid reagent for uric acid'.

3. The concentration of ergothioneine in the vesicular secretion was determined in samples from

twenty boars. The contents ranged from 29 to 256 mg./100 ml.; the average was 79 mg./100 ml.

4. From 1300 ml. of boar vesicular secretion 0·48 g. pure crystalline ergothioneine has been isolated. The product has been identified by elementary analysis, the determination of the bromine-oxidizable sulphur, diazo reaction, reducing properties, and by the preparation of certain derivatives including the crystalline mercury compound.

5. For the detection of small amounts of ergothioneine in tissues, urine and other body fluids, a chromatographic method has been developed, which depends on the localization of ultraviolet-absorbing areas on paper chromatograms followed by spraying with the diazo reagent or certain other colour-yielding reagents. In this manner it was possible to detect less than 5 μg. ergothioneine, and to distinguish ergothioneine from thiolhistidine, uric acid and ascorbic acid.

6. Thiolhistidine, either free or bound, does not accompany ergothioneine in the boar seminal vesicles or in the vesicular secretion. The content of ascorbic acid in the boar vesicular secretion was low; in bull, on the other hand, there was little ergothioneine, and the high reducing value of the vesicular secretion was largely due to ascorbic acid. Ram and human semen showed the presence of some reducing and diazo-reactive substances which, though not identical, may possibly be related to ergothioneine.

7. Boar urine, unlike boar semen, contained only a negligible amount of ergothioneine. The claim that human urine contains ergothioneine has not been confirmed. No ergothioneine, or only a trace of it, was found in pig foetal fluids, vitreous fluid of the eye, corpora lutea, adrenal glands and thyroid gland.

8. Ergothioneine, in a concentration corresponding to that found in the boar vesicular secretion, protected spermatozoa against the action of sulphydryl inhibitors. Both motility and fructolysis, under anaerobic as well as aerobic conditions, were abolished in sperm suspensions by 10^{-3} M-iodosobenzoic acid, but could be maintained at a normal level if 2×10^{-3} M-ergothioneine was also added. Ergothioneine also counteracted the inhibition caused by cupric ions and hydrogen peroxide.

9. The protective effect of ergothioneine against inhibitors of SH-groups was not limited to sperm but could be demonstrated in muscle glycogenolysis and yeast fermentation.

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Quantitative Paper Chromatography of Reducing Steroids of the Adrenal Cortex

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The methods commonly employed for the determination of steroids furnish only limited information when applied to adrenal extracts. Not only is it impossible to estimate individual members of the group of biologically active corticoids in a mixture, but the presence of many closely related, but inactive, compounds in gland extracts renders the overall determination of reducing or formaldehydogenic activity largely valueless. Since paper chromatography affords a ready means of resolving the complex mixture of cortical steroids into single compounds or groups of closely related compounds, the quantitative evaluation of particular members of the cortical group thus becomes feasible.

It was desired to develop a method which would enable the steroids to be located on the chromatogram and to be determined colorimetrically by elution of the product of the reaction, thus avoiding the necessity of running reference strips or relying on the R_F values for the elution of the appropriate portions. A survey of the known reactions of steroids applicable to paper chromatograms indicated that they either lacked sensitivity or that they were not quantitative. The arsenomolybdate reaction reported previously (Schwarz, 1952*a*) was found to be satisfactory in both respects. The technique described below is simple and does not require any elaborate equipment. It readily lends itself to routine analysis of mixtures of cortical steroids and it should prove useful for the determination of reducing cortical steroids in blood and urine.

EXPERIMENTAL

Chromatography

The solvent systems described by Zaffaroni, Burton & Keutmann (1950) and by Bush (1952) have been used. With the former the more polar and the less polar steroids have to be resolved on separate strips, one being run in toluene/propylene glycol, the other in benzene/formamide. The time required for satisfactory separation depends on a number of factors, but it is reasonably constant under strictly standardized conditions. In the experience of the author, 6–7 hr. development with benzene of the formamide-impregnated strips gives a separation of the fast-running steroids satisfactory for quantitative purposes, while a running time of about 100 hr. in toluene ensures a reasonable separation of hydrocortisone (17-hydroxycorticosterone) from very polar material at the top of the chromatogram.

Several of the solvent systems described by Bush (1952) enable all the biologically active steroids to be separated from each other on one strip and in a much shorter time. The practical difficulties associated with chromatographing at 34° have been overcome by the use of the 'chromatocoil' (Schwarz, 1952*b*), in which the paper strip is accommodated in spiral form in a container which can readily be placed in an incubator. A paper strip 50 cm. in length, or longer, can easily be fitted into an apparatus measuring no more than 5 cm. diameter × 10 cm. long. The use of the ascending technique has the advantage that development stops automatically when the solvent front reaches the end of the strip, thus making accurate timing unnecessary. Moreover, with the same length of strip, the steroids will always travel approximately the same distance. While chromatographic development is somewhat slower than in the descending arrangement, saturation is effected much more quickly owing to the dimensions of the apparatus. Thus a complete run can be carried out in 5–6 hr.