

## Further Studies on Seminal Ergothioneine of the Pig

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In a previous paper (Heath, Rimington, Glover, Mann & Leone, 1953) it was concluded that the mature boar was capable of utilizing the sulphur of methionine for the synthesis of ergothioneine but not that of inorganic sulphate or thiolhistidine. Recent work by Melville, Otken & Kovalenko (1955) showed that, in the immature pig, methionine sulphur does not act as a source of ergothioneine sulphur. In order to clarify the situation it was decided to repeat the earlier experiments. The present paper reports the results of this study, together with some new observations on the incorporation of radioactive sulphur administered as [<sup>35</sup>S]methionine into spermatozoa and seminal plasma of the boar.

### MATERIALS AND METHODS

*Experimental animal and diet.* The animal used in the present study was a boar from an inbred strain of 'large whites' maintained at the Animal Research Station. The boar was 7 years' old and weighed 300 kg. It was fed twice daily, the daily ration consisting of approx. 3.5 kg. of a feed mixture containing 40% of middlings (wheat), 50% of barley meal, 12% of fish meal, 8% of lucerne meal and 2% of a mineral mixture. Regular removal of faeces was carried out and care was taken to avoid contamination of feeding-stuff with excreta.

*Collection of semen and blood.* The procedure for the collection of boar semen was that of Wallace (1949); the artificial vagina was a modification of that used by Ito, Niwa & Kudo (1948), as described by Glover & Mann (1954). The volume of the semen varied from 350 to 560 ml./collection. After removal of the seminal gel by filtering through a Büchner funnel (without paper), the filtrate was centrifuged at 1000 g to separate the spermatozoa from the seminal plasma. The spermatozoa were washed twice with 5 vol. of Ringer solution, and the washing fluids added to the seminal plasma. Blood was collected from the auricular vein, in the presence of heparin.

*Fractionation of seminal plasma and sperm.* The sperm and seminal plasma separated by the method outlined above were further fractionated.

The spermatozoa, after washing with Ringer solution, were separated by grinding in ethanol (5 vol.), centrifuged after 1 hr., re-extracted with ethanol (5 vol.), then with ethanol-ether (3:1) and finally with ether. In this manner two fractions were obtained from the sperm, the ethanol-ether-soluble portion, which represents chiefly sperm lipids,

and the ethanol-ether-insoluble portion, which consists mainly of deoxyribonucleoprotein and other intracellular sperm proteins (cf. Mann, 1954). Of these two fractions, the first was concentrated *in vacuo* to one-twentieth of the original volume, whereas the ethanol-ether-insoluble protein fraction was dried, yielding 1-3 g. of powder/ejaculate.

The seminal plasma was treated with ethanol (4 vol.), centrifuged after 1 hr. and thus separated into the ethanol-soluble portion, which contains ergothioneine (Mann & Leone, 1953), and the ethanol-insoluble protein fraction. The ethanol extract from the seminal plasma was concentrated *in vacuo* at pH 6 to one-tenth of the original volume of semen, whereas the ethanol-insoluble protein fraction was washed first with ethanol and then with ether, yielding 5-15 g. of powder/ejaculate.

*Fractionation of sulphur compounds in seminal plasma.* The method of Heath *et al.* (1953) was used.

*Administration of [<sup>35</sup>S]methionine.* DL-[<sup>35</sup>S]Methionine (63 mg., 5.5 mc) was obtained from the Radiochemical Centre, Amersham. The radioactive material was dissolved in 300 ml. of water and administered to the boar by adding 100 ml. of the solution to the daily food ration, on three consecutive days. Care was taken to ensure that all food was eaten by the animal.

*Radioactivity measurements.* Samples were counted in 1 cm.<sup>2</sup> planchets, with a thin mica end-window Geiger tube. Samples which gave low or zero activities were subsequently measured with a windowless gas-flow counter, constructed as described by Garrow & Piper (1955).

*Ergothioneine estimations.* These were made by the method of Hunter (1949).

*Ion-exchange resin.* Zeo-Karb 225, 4½% cross-linked, 100-150 mesh, was obtained from the Permutit Co., London. Ergothioneine can be quantitatively eluted from this resin with 0.1 N-NH<sub>3</sub> soln. in a small sharp band; with higher degrees of cross-linkage, ergothioneine is not quantitatively eluted and the elution band is broad. With the 4½% cross-linked resin, ergothioneine is displaced by 0.1 N-NH<sub>3</sub> soln. immediately before the acidic amino acids and not, as one would expect, with the basic amino acids.

*Ethanol purification.* The ethanol used for the alumina chromatography was purified by distillation from magnesium and iodine. The distillate was redistilled from zinc.

### *Isolation of ergothioneine from seminal plasma*

*Extraction with ethanol.* Seminal plasma (390 ml.), obtained from semen (480 ml.) as described above, was deproteinized with ethanol (1.6 l.), and the filtrate, adjusted to pH 6 with acetic acid, was concentrated under reduced

pressure to 40 ml. Ethanol (200 ml.) was added and, after standing at  $-10^{\circ}$  for 2 hr. and centrifuging, the clear, pale-yellow supernatant was concentrated under reduced pressure almost to dryness. The residue was dissolved in water and made up to 250 ml. Suitable portions of this solution were used for ergothioneine estimation.

*Ion-exchange chromatography.* The method of Westall (1952) yielded an ergothioneine-rich fraction suitable for secondary purification by alumina chromatography (Melville, Horner & Lubschez, 1954). The solution described above was passed through a series of three columns (20 cm.  $\times$  1 cm.; 15 cm.  $\times$  1 cm.; 5 cm.  $\times$  0.8 cm.) of the Zeo-Karb 225, in the  $H^+$  form. The columns were washed with water (100 ml.) and then with 0.1N- $NH_3$  soln., the progress of displacement being followed by the change in colour of the resin. Fractions (5 ml.) were collected and one drop of each fraction was tested on filter paper for the presence of ergothioneine with 0.2% (w/v) ethanolic 2:6-dichloroquinonechloroimide (British Drug Houses Ltd.). A brick-red colour, which appears as soon as the ethanol evaporates, indicates the presence of ergothioneine; some aromatic compounds give a grey colour which appears more slowly. The fractions were also tested with ninhydrin. The fractions containing ergothioneine were combined and concentrated under reduced pressure nearly to dryness. The residue was transferred to a small glass dish and drying was completed over  $H_2SO_4$ .

*Alumina chromatography.* Activated alumina (type 'H', Peter Spence and Sons, Widnes), 100–150 mesh, was washed four times by decantation with a mixture of purified ethanol (300 ml.), water (100 ml.) and formic acid (4 ml.). A column (30 cm.  $\times$  1 cm.) was prepared from the resulting slurry. The total residue containing ergothioneine was transferred to the column with the minimum amount of the aqueous ethanol-formic acid mixture, and the column was then developed with the same solvent, 2 ml. fractions being collected. Each fraction was tested, as described above, for the presence of ergothioneine and amino acids. Ergothioneine was eluted in the fractions representing 20–50 ml. of solvent flow, whereas the amino acids were not eluted before 180 ml. of solvent had been passed. All fractions containing ergothioneine were combined and evaporated to dryness. In order to convert the ergothioneine into the free base and remove traces of aluminium compounds, the residue was dissolved in water and chromatographed on a 5 cm.  $\times$  0.8 cm. column of the Zeo-Karb 225  $H^+$  form. After washing with water, 0.1N- $NH_3$  soln. was applied and the fractions containing ergothioneine were collected, combined and evaporated to dryness.

The dried residue was exhaustively extracted with cold dry ethanol and the crystals of insoluble ergothioneine were filtered off and washed with ethanol and ether. The ergothioneine (65 mg.) thus obtained was dissolved in water (2 ml.) and filtered. The filtrate and washings were concentrated to 2 ml. and tartaric acid (40 mg.) was added to the hot solution. On cooling, crystallization rapidly occurred. The resulting ergothioneine tartrate was recrystallized from aqueous ethanol (wt. 74 mg.).

*Criteria of purity of ergothioneine tartrate.* Melting point  $235^{\circ}$ ; mixed m.p. with ergothioneine tartrate,  $235^{\circ}$ . (Melting points were determined on a Gallenkamp electrically heated micro-melting-point apparatus, and were uncorrected.) When analysed by the method of Hunter (1949) the isolated ergothioneine tartrate was shown to be 100%

pure. By one-dimensional chromatography in butanol-acetic acid-water (60:15:25, by vol.) and treatment with dichloroquinonechloroimide, only one substance was revealed corresponding to ergothioneine ( $R_f$  0.29); no ninhydrin-reacting substances were present.

#### *Isolation of ergothioneine from blood*

Red blood corpuscles separated by centrifuging from 350 ml. of heparinized blood were washed twice with 350 ml. of isotonic saline solution, lysed with an equal volume of water, and extracted with cold ethanol and  $CHCl_3$  by the procedure of Mann & Keilin (1937). The haemoglobin-free extract thus obtained was concentrated *in vacuo* to remove ethanol. This solution (214 ml.) contained 7.7 mg. of ergothioneine (equivalent to 2.2 mg./100 ml. of whole blood). The procedure for the isolation of ergothioneine was the same as for the isolation from semen; wt. of recrystallized ergothioneine tartrate 6.8 mg.; m.p.  $235^{\circ}$ .

## RESULTS

### *Experiments on the ethanol-soluble portion of seminal plasma*

Details of the semen collections, volumes and the ergothioneine contents of seminal plasma are given in Table 1. Crystalline ergothioneine tartrate was isolated separately, by the procedure described above, from collections nos. I–V, and from a representative sample of the bulked collections nos. VI–XI. Each sample was rigorously purified and tested quantitatively by the diazo method of Hunter and by melting point and paper chromatography to establish purity and identity of the product. The samples were then 'counted' either as ergothioneine tartrate or after oxidation with bromine-water, as barium sulphate, at infinite thickness in 1 cm.<sup>2</sup> planchets, both with the end-window Geiger tube and the windowless gas-flow counter. In no case was any detectable radioactivity above background observed, even on prolonged counting.

Portions of the ethanol extracts of seminal plasma, collections nos. II–V, were combined and then separated into two fractions representing the free plus ethereal sulphates and the bromine-oxidizable sulphur respectively. The barium sulphate samples thus isolated were 'counted' at a thickness of 2 mg./cm.<sup>2</sup> rather than at infinite thickness, owing to the small amount of material obtained from the free plus ethereal sulphate fraction. The total activities were 141 counts/min. for the free plus ethereal sulphate fraction and 420 counts/min. for the bromine-oxidizable sulphur fraction. The amount of barium sulphate isolated from the bromine oxidation was equivalent to 95% of the ergothioneine present, as determined by the Hunter diazo test. The method of isolation was almost quantitative and as the ergothioneine after isolation proved to be inactive (see above), the

Table 1. *Data on collections of boar semen and the ergothioneine content of seminal plasma*

Before the administration of radioactive methionine, three collections were made, A, B and C, and of these A was analysed for ergothioneine. The radioactive compound, 5.5 mc of DL- $^{35}\text{S}$ methionine (63 mg.), was dissolved in 300 ml. of water and 100 ml. fed to the animal on three consecutive days, beginning 31 May 1955.

No.	Date	Time after last feeding of $^{35}\text{S}$ (days)	Vol. of seminal plasma (ml.)	Ergothioneine (mg./100 ml. of seminal plasma)	Total ergothioneine (mg.)
A	17. v. 55	—	270	33.3	90.0
B	26. v. 55	—	—	—	—
C	31. v. 55	—	300	—	—
I	6. vi. 55	4	300	24.4	73.1
II	10. vi. 55	8	350	12.7	44.6
III	13. vi. 55	11	310	13.5	42.0
IV	18. vi. 55	16	360	11.1	40.0
V	21. vi. 55	19	360	12.5	45.0
VI	27. vi. 55	25	235	9.5	22.4
VII	4. vii. 55	32	350	9.1	32.0
VIII	11. vii. 55	39	305	7.8	23.8
IX	18. vii. 55	46	325	7.6	24.6
X	8. viii. 55	67	340	8.1	27.6
XI	24. viii. 55	83	370	7.0	25.5

radioactivity of the bromine-oxidizable sulphur fraction must reside in a small quantity of a relatively highly labelled substance.

#### *Experiments on the ethanol-insoluble fraction of seminal plasma*

As can be seen from Table 1, the semen collections were made from 4 to 83 days after the administration of 5.5 mc of  $^{35}\text{S}$ methionine, and in order to determine the general level of labelling of the body sulphur, separate samples of the ethanol-precipitated protein from the seminal plasma of collections nos. I and XI were oxidized (Heath *et al.* 1953) and the sulphur was isolated as barium sulphate. At the times of collection the respective counts of seminal plasma protein I and XI were 2608 and 325 counts/min. at infinite thickness and 1 cm.<sup>2</sup> area by the end-window tube. It is apparent that the general level of labelling of the sulphur in the seminal plasma protein was sufficiently high during the whole course of the experiment for radioactivity to have been detected in the isolated ergothioneine if biosynthesis from cystine or methionine had taken place.

#### *Experiments with blood*

Ergothioneine was isolated from a sample of blood withdrawn 49 days after the administration of the radioactive methionine. This was counted as ergothioneine tartrate (6 mg./cm.<sup>2</sup>) but no activity above background was observed. A portion of this blood plasma was oxidized and the sulphur isolated as barium sulphate; it had an activity of 538 counts/min./cm.<sup>2</sup> at infinite thickness when counted with the end-window tube.

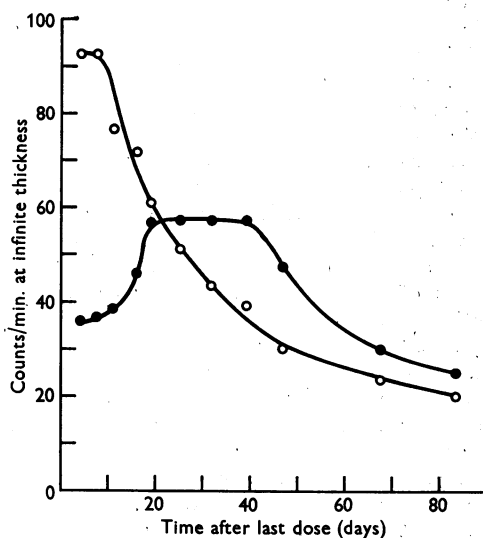


Fig. 1. Radioactivity of  $\text{BaSO}_4$  prepared by oxidation of the total sulphur in boar seminal-plasma protein (O) and sperm protein (●) obtained after feeding 5.5 mc of DL- $^{35}\text{S}$ methionine.

#### *Experiments to determine the time required for the appearance of radioactive sulphur in the proteins of ejaculated spermatozoa and seminal plasma*

As shown previously, after feeding  $^{35}\text{S}$ methionine to a boar, the isotope appears in the proteins of the seminal plasma much sooner than in the ejaculated spermatozoa (Heath *et al.* 1953). This observation was extended by further studies, the results of which are illustrated in Fig. 1. At various time intervals, ranging from 4 to 83 days after the

administration of [ $^{35}\text{S}$ ]methionine, the proteins were separated from the sperm and seminal plasma of ten collections, and radioactivity measurements carried out by counting at infinite thickness after the main experiment was completed. All counts were made on the same day so that decay corrections were not necessary. As can be seen from Fig. 1, the seminal-plasma proteins of ejaculated boar semen show a high degree of labelling within a few days of feeding [ $^{35}\text{S}$ ]methionine, and soon afterwards the radioactivity declines sharply. In contrast to the seminal plasma, the spermatozoa show only a low activity during the first 2 weeks after the administration of radioactive sulphur and attain a high level much later, namely, during the period between 20 and 40 days after feeding.

### DISCUSSION

By an improved technique, which is generally applicable to other complex mixtures, pure crystalline ergothioneine was isolated from eleven collections of semen, taken from 4 to 83 days after the administration of 5.5 mc of DL- $^{35}\text{S}$ ]methionine to a 7-year-old boar weighing 300 kg. In no case was any detectable radioactivity observed in the isolated ergothioneine. Ergothioneine was also isolated from the blood 49 days after the feeding of the isotope, and again the ergothioneine was not demonstrably radioactive. On the other hand, the general labelling of the sulphur of both the seminal plasma and the blood plasma at all times when samples of semen were taken for ergothioneine isolation was high enough, even for a 300 kg. experimental animal, for radioactivity to have been detected in the isolated ergothioneine if either direct biosynthesis or even a dynamic interchange of sulphur between ingested ergothioneine and other radioactive sulphur compounds had taken place. When viewed with respect to the dynamic state of other body constituents such as glutathione, which in the erythrocytes of normal man has a half-life of approximately 4 days (Dimant, Landsberg & London, 1955), the biochemical inertness of ergothioneine in such vital body fluids as blood and semen is remarkable. Dietary ergothioneine is known to be incorporated slowly into blood, where it occurs intracellularly (Heath, Rimington, Searle & Lawson, 1952), and rapidly into the seminal plasma, which is an extracellular fluid (Heath *et al.* 1953). As no ergothioneine is detectable in blood plasma the possibility could be envisaged that one source of seminal ergothioneine might be ergothioneine released from the erythrocytes as a result of their breakdown. Bush *et al.* (1955) have shown that the mean red-cell survival time in pigs is 62 days. In the present study no radioactivity was found in the ergothioneine isolated from the blood

49 days after the administration of radioactive isotope and from semen up to 83 days. Therefore the possibility that the site of biosynthesis of ergothioneine is connected with the erythropoietic system and that seminal ergothioneine is at least in part derived from the breakdown of matured erythrocytes appears improbable.

Under the conditions of the present experiment biosynthesis of ergothioneine did not occur. This experiment differed from our previous experiment (Heath *et al.* 1953) in that certain cereals such as oats and maize were rigorously excluded from the diet. It has been established (Baldrige, 1955; Melville *et al.* 1955) that these foodstuffs do increase blood ergothioneine levels. Recently Melville & Eich (1956) isolated ergothioneine from a sample of commercial rolled oats. The whole history of the investigations of the source of ergothioneine has been full of contradictions. We have shown that synthetically prepared [ $^{35}\text{S}$ ]ergothioneine, when fed to the boar, passes readily into the seminal plasma (Heath *et al.* 1953). Melville *et al.* (1955) have also shown that there are variations in the uptake of added dietary ergothioneine by rats and that zein adversely affects this uptake. Fraser (1951) has shown that there are marked variations in blood ergothioneine levels in persons of different race maintained on the same hospital diet for over 3 months; also rapid changes can occur after injury. Although the results presented here seem most adequately to confirm that biosynthesis of ergothioneine does not take place in the pig, we feel that any conclusion should be drawn with caution. Even if ergothioneine were not present itself in a diet, one or other dietary component might supply an essential precursor for the biosynthesis of ergothioneine in the body, and this contingency has to be reckoned with in interpreting experimental results.

The present investigation was conducted under better experimental conditions than our previous work (Heath *et al.* 1953); not only was the administered radioactivity more than five times greater but instead of relying upon sulphur fractionation, the ergothioneine was in all cases isolated as such and recrystallized to zero activity. The present results therefore confirm the work of Melville *et al.* (1955) and support the hypothesis that ergothioneine is not biosynthesized in the animal body. At the same time, our results point to the existence in boar semen of another compound containing sulphur which is oxidized to sulphate by bromine water. This is clearly established by the labelling of the bromine-oxidizable sulphur fraction after the administration of [ $^{35}\text{S}$ ]methionine. It seems improbable that the unknown compound is either sulphide or sulphite, as the method of hydrolysis of ethereal sulphates involves boiling in acid solution

for 30 min. The use of bromine-oxidizable sulphur as a method of estimation (Touster, 1951) or fractionation (Heath *et al.* 1953) of ergothioneine can lead to erroneous conclusions. Unidentified sulphur compounds have recently been detected in blood by Smith & Tuller (1955).

The peculiar conditions which govern the occurrence and distribution of ergothioneine in semen and male accessory secretions certainly point to the existence of factors other than dietary. Ergothioneine, it should be noted, is secreted in the male reproductive tract only in certain well-defined glandular structures. Whereas in the boar, for instance, it is confined to the seminal vesicle and does not occur in the Cowper's gland, epididymis or elsewhere, in the stallion it originates in the ampullae (Leone, 1954; Mann, Leone & Polge, 1956). It is also worth noting that ergothioneine has been recently shown to occur in large concentrations in the male accessory secretions of two Insectivora, namely, the mole and the hedgehog (Mann, 1956). On the other hand, the semen of man, ram and bull appears to contain only negligible quantities of this base.

It has been shown that in ejaculated boar semen maximum radioactivity appears much earlier in the seminal plasma than in the spermatozoa. The delay with which radioactive sulphur appears in ejaculated spermatozoa is undoubtedly due to the fact that the processes of spermatogenesis, sperm maturation and sperm transport in the male reproductive tract require much more time than the secretory processes in the male accessory organs and the formation of seminal plasma.

#### SUMMARY

1. A new, generally applicable method of isolating pure ergothioneine from biological material is described.

2. DL-[<sup>35</sup>S]Methionine (5.5 mc) was administered to a mature pig; no radioactivity could be detected in the blood ergothioneine after 49 days or in the seminal ergothioneine up to 83 days after the administration of radioactive isotope.

3. Under the feeding conditions of this experiment no biosynthesis or metabolic exchange of ergothioneine sulphur with other labelled body constituents took place.

4. An unidentified sulphur compound which is oxidizable to sulphate by bromine water is present in pig semen.

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#### REFERENCES

- Baldrige, R. C. (1955). *J. Nutr.* **56**, 107.  
 Bush, J. A., Berlin, N. I., Jensen, W. N., Brill, A. B., Cartwright, G. E. & Wintrobe, M. M. (1955). *J. exp. Med.* **101**, 451.  
 Dimant, E., Landsberg, E. & London, I. M. (1955). *J. biol. Chem.* **213**, 769.  
 Fraser, R. S. (1951). *J. Lab. clin. Med.* **37**, 199.  
 Garrow, J. & Piper, E. A. (1955). *Biochem. J.* **60**, 527.  
 Glover, T. & Mann, T. (1954). *J. agric. Sci.* **44**, 355.  
 Heath, H., Rimington, C., Glover, T., Mann, T. & Leone, E. (1953). *Biochem. J.* **54**, 606.  
 Heath, H., Rimington, C., Searle, C. E. & Lawson, A. (1952). *Biochem. J.* **50**, 530.  
 Hunter, G. (1949). *Canad. J. Res.* **27**, 230.  
 Ito, S., Niwa, T. & Kudo, A. (1948). *Bull. zootech. Exp. Sta. Chiba-Shi*, **55**, 1.  
 Leone, E. (1954). *Nature, Lond.*, **174**, 404.  
 Mann, T. (1954). *The Biochemistry of Semen*. London: Methuen.  
 Mann, T. (1956). *Recent Progr. Hormone Res.* **12**, 353.  
 Mann, T. & Keilin, D. (1937). *Proc. Roy. Soc. B*, **122**, 119.  
 Mann, T. & Leone, E. (1953). *Biochem. J.* **53**, 140.  
 Mann, T., Leone, E. & Polge, C. (1956). *J. Endocrin.* **13**, 279.  
 Melville, D. B. & Eich, S. (1956). *J. biol. Chem.* **213**, 647.  
 Melville, D. B., Horner, W. H. & Lubschez, R. (1954). *J. biol. Chem.* **206**, 221.  
 Melville, D. B., Otken, C. C. & Kovalenko, V. (1955). *J. biol. Chem.* **216**, 325.  
 Smith, E. L. & Tuller, E. F. (1955). *Arch. Biochem. Biophys.* **54**, 114.  
 Touster, O. (1951). *J. biol. Chem.* **188**, 371.  
 Wallace, C. (1949). *J. Endocrin.* **6**, 205.  
 Westall, R. G. (1952). *Biochem. J.* **52**, 638.