Studies Using Radioactive Sulphur on Ergothioneine Formation in the Pig

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(Received 14 January 1953)

Until recently, blood erythrocytes were the only known source of ergothioneine in the animal body, and so far all studies on the formation of ergothioneine have been confined to blood. In experiments on the pig, rabbit and rat it has been found that certain diets, although themselves devoid of ergothioneine, can influence the level of ergothioneine in the blood (Eagles & Vars, 1928; Potter & Franke, 1935; Hunter, 1951; Spicer, Wooley & Kessler, 1951). Ergothioneine added to the diet was found to be incorporated into the blood of rats and rabbits (Spicer *et al.* 1951; Heath, Rimington, Searle & Lawson, 1952) and synthetic [³⁵S]ergothioneine fed to rats was shown by Heath (1953) to find its way into the blood and tissues.

Leone & Mann (1951) and Mann & Leone (1953) recently brought to light a second source of ergothioneine in the animal body, when they discovered that ergothioneine is a normal constituent of boar semen, and is secreted by the seminal vesicles. Unlike blood, the vesicular secretion was shown to contain ergothioneine as an extracellular constituent and in a remarkably high concentration.

The present paper reports the results of studies on the formation of both seminal and blood ergothioneine, obtained after feeding to boars ³⁵S-labelled inorganic sulphate, thiolhistidine, methionine and ergothioneine. It was found that neither [³⁵S]sulphate nor [³⁵S]thiolhistidine acts as a source of sulphur for ergothioneine. On the other hand, both [³⁵S]methionine and [³⁵S]ergothioneine were shown to be capable of labelling the body ergothioneine.

MATERIALS AND METHODS

Experimental animals. Two boars were used, A and B, both from an inbred strain of 'Large Whites' maintained at the Animal Research Station, Cambridge. At the start of the experiment, boar A was 3 years old, and weighed 220 kg. and

boar B was 1 year old and weighed 150 kg. The boars were fed twice daily, the daily ration consisting of approximately 3 kg. of balanced meal mixture. Regular removal of faeces was carried out, and particular care taken at all times to avoid contamination of food with excreta.

Collection of semen, blood and urine. The semen was collected by means of an artificial vagina (Walton, 1945), usually at weekly intervals. As a rule, one complete ejaculate was collected on each occasion. The volume of the ejaculates varied from 200 to 600 ml., the density of semen from 50 000 to 200 000 sperm/μl. After removal of the gel, the semen was separated by centrifugation into sperm and seminal plasma, the sperm washed with Ringer solution, and the washing fluid added to the seminal plasma. Both the sperm and the seminal plasma were then treated with 3 vol. of ethanol, centrifuged after 1 hr., and thus separated into fractions, referred to hereafter as the ethanol-insoluble and ethanol-soluble portions; ergothioneine is confined entirely to the ethanol-soluble portion of the seminal plasma (Mann & Leone, 1953). The ethanolic extracts, both from the sperm and the seminal plasma, were concentrated *in vacuo*, at pH 6, to one-tenth of the original volume of semen. The ethanol-insoluble portions from sperm and plasma were washed with ethanol and dried.

Blood was collected from the auricular vein, in the presence of heparin. Urine was collected into a glass vessel placed outside the pen and connected by a rubber tube to a plastic funnel enclosing the prepuce and attached to the body by means of a plastic apron. In this manner, it was possible to obtain clean urine.

³⁵S-labelled compounds and their administration. H₂³⁵SO₄ and [³⁵S]methionine were obtained from the Atomic Energy Research Establishment, Harwell, and the Radiochemical Centre, Amersham. [³⁵S]Thiolhistidine and [³⁵S]ergothioneine were synthesized by the method of Heath, Lawson & Rimington (1951) using 25 g. KCN³⁵S containing 10 mc. ³⁵S obtained from the Radiochemical Centre, Amersham. The radioactive compounds were administered to the boars in the form of dilute aqueous solutions added to their food. Care was taken to ensure that all the food was eaten by the animals.

Determination of total ³⁵S in tissues. The following quantities were used for wet oxidation: whole blood, 2 ml.; blood plasma, 5 ml.; blood cells, previously washed seven times with 0.9% (w/v) NaCl, 4 ml.; seminal plasma, ethanol-soluble portion, 3 ml., ethanol-insoluble portion, 0.5 g.; sperm, ethanol-soluble portion 5 ml., ethanol-insoluble portion

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0.5 g.; urine 6 ml. All samples were refluxed for 18 hr. with 15 ml. of a mixture of 1 vol. 60% (w/v) HClO_3 + 3 vol. 70% (w/v) HNO_3 in 50 ml. Kjeldahl flasks containing 0.1 g. Cu wire. The solutions were evaporated to dryness, the residue redissolved in 4 ml. 6N-HCl and again evaporated to dryness, redissolved in 15 ml. boiling water with addition (if necessary to effect complete solution) of 0.1 ml. 6N-HCl. Carrier H_2SO_4 ($\equiv$ 10 mg. BaSO_4) was added to the solutions obtained from ethanol-soluble portions of seminal plasma and semen. The total sulphate ions were then precipitated as BaSO_4 , collected and washed in tared 10 ml. centrifuge tubes and, after drying at 105° and weighing, samples were transferred with 50% (v/v) ethanol to tared nickel planchets. The activity was determined with an end-window counter.

Fractionation of urinary sulphur

Boar urine is normally devoid of ergothioneine (Mann & Leone, 1953). However, as a result of feeding, particularly with [^{35}S]ergothioneine, it became necessary to include determinations of bromine-oxidizable S in the urine. The fractionation was carried out as follows:

Free sulphate. The free sulphate was precipitated as BaSO_4 from 10 ml. urine.

Ethereal sulphate. Urine (50 ml.) was treated with 20 ml. 3N-HCl and 10 ml. 5% (w/v) BaCl_2 solution and filtered through a Whatman no. 42 filter paper. Carrier H_2SO_4 ($\equiv$ 25 mg. BaSO_4) was added to the filtrate, and the mixture boiled for 30 min. The BaSO_4 was collected as described above for total S.

Bromine-oxidizable S. To the supernatant liquid, filtered through a Whatman no. 42 filter paper, were added carrier H_2SO_4 ($\equiv$ 25 mg. BaSO_4) and 2 ml. Br_2 ; after 30 min., excess Br_2 was removed by boiling and the BaSO_4 collected as previously described.

All the BaSO_4 fractions obtained from urine were washed with 2N-NaOH.

Fractionation of sulphur in seminal plasma

As shown by Mann & Leone (1953), the bulk of the non-protein sulphur present in the vesicular secretion of the boar can be accounted for as ergothioneine. In the whole semen, however, as ejaculated, the vesicular secretion constitutes no more than 25% of the total volume (McKenzie, Miller & Bauguess, 1938), the remaining part being made up by sperm and various secretory fluids including those of epididymis, prostate and Cowper's glands. It was therefore necessary to carry out a complete analysis of the various sulphur fractions.

Free and ethereal sulphate. To the ethanol-soluble portion of seminal plasma (15 ml.) were added 35 ml. carrier H_2SO_4 ($\equiv$ 25 mg. BaSO_4), 10 ml. 3N-HCl and 5 ml. 5% (w/v) BaCl_2 and the mixture was boiled for 30 min. The BaSO_4 was collected as described above.

Bromine-oxidizable S. The supernatant liquid thus obtained was filtered through a Whatman no. 42 paper, and treated with Br_2 as described for the corresponding determination in urine. In this case the addition of carrier H_2SO_4 was unnecessary.

RESULTS

Experiments with $\text{H}_2^{35}\text{SO}_4$

Before carrying out the actual feeding experiment, proof had to be obtained that $\text{H}_2^{35}\text{SO}_4$ added to

ejaculated semen *in vitro* does not confer any radioactive properties on seminal ergothioneine. A sample (1 ml.) of a solution containing $0.05 \mu\text{c}$. $\text{H}_2^{35}\text{SO}_4$ was added to 90 ml. boar semen, the mixture was centrifuged 6 hr. later, and the sperm and seminal plasma fractionated into the ethanol-soluble and ethanol-insoluble portions. As can be seen from Table 1, some $^{35}\text{SO}_4$ was carried down with the ethanol-insoluble portions of both sperm and seminal plasma, but the bromine-oxidizable sulphur fraction was completely free from radioactivity. An additional control experiment was carried out in which a solution containing a mixture of pure ergothioneine and $\text{H}_2^{35}\text{SO}_4$ was separated into inorganic and bromine-oxidizable sulphur fractions. The latter was not radioactive (Table 1).

Table 1. Radioactivity of BaSO_4 prepared by the fractionation of (a) a mixture of boar semen and $\text{H}_2^{35}\text{SO}_4$ and (b) a mixture of ergothioneine and $\text{H}_2^{35}\text{SO}_4$

	Counts
(a) Seminal plasma	
Ethanol insoluble. Total S	31
Ethanol soluble. Total S	322
Ethanol soluble. Free + ethereal sulphate	1027
Ethanol soluble. Bromine-oxidizable S	0
(b) Sperm	
Ethanol insoluble. Total S	41
Free + ethereal sulphate	457
Bromine-oxidizable S	0

Two experiments were carried out on boars fed with inorganic ^{35}S -labelled sulphate. In the first experiment, on boar A, 10 mc. carrier-free $\text{H}_2^{35}\text{SO}_4$ were diluted with water to 200 ml., and 50 ml. added to the daily ration on 4 successive days. Semen, blood and urine were then collected at weekly intervals. The results are recorded in Table 2.

It will be seen that the relatively high radioactivity of the free sulphate of urine and seminal plasma falls rapidly during the first 2 weeks. The slight radioactivity in the bromine-oxidizable fraction of the seminal plasma may be due not to ergothioneine but to possible traces of sulphide, thiosulphate or thiocyanate, although none of these was detectable by normal chemical means. The weight of barium sulphate obtained agreed with the calculated amount based on the colorimetric determination of ergothioneine; nevertheless, even a trace of the above-mentioned compounds with labelling comparable with that of the free sulphate fraction could account for the activity observed.

To decide whether or not the slight activity was due to ergothioneine, a second experiment was carried out on boar B in which 10 mc. carrier-free $\text{H}_2^{35}\text{SO}_4$ was diluted with water to 1 l. and 50 ml. of

Table 2. *Radioactivity of BaSO₄ prepared by the fractionation of the sulphur-containing constituents of boar urine, blood and semen obtained after feeding 10 mc. H₂³⁵SO₄*

(Values are expressed as counts/min. in 1 sq.cm. sample of 'infinite thickness'.)

	Days after last dose				
	3	10	17	24	31
	Counts				
Seminal plasma					
Ethanol insoluble. Total S	66	55	41	26	19
Ethanol soluble. Total S	400	211	77	74	55
Ethanol soluble. Free + ethereal sulphate	2350	1052	402	281	196
Ethanol soluble. Bromine-oxidizable S	105	69	60	53	53
Sperm					
Ethanol insoluble. Total S	—	45	51	37	32
Ethanol soluble. Total S	—	192	128	61	56
Blood					
Total S	330	83	47	25	18
Plasma. Total S	—	156	63	31	30
Cells. Total S	—	38	26	11	14
Urine					
Total S	5200	679	238	201	98
Free sulphate	5700	805	271	246	149
Ethereal sulphate	—	800	310	262	140
Bromine-oxidizable S	—	72	27	95	50

the solution administered daily on 20 successive days, so as to maintain a high level of ³⁵SO₄²⁻ activity in the animal for a longer period than in the first experiment. The results are set out in Table 3.

Table 3. *Radioactivity of BaSO₄ prepared by the fractionation of the sulphur containing constituents of boar semen obtained after the feeding of 10 mc. H₂³⁵SO₄ spread over a period of 20 days*

(Values are expressed as counts/min. in 1 sq.cm. sample of 'infinite thickness'.)

	Seminal plasma (counts)	Sperma- tozoa (counts)
Ethanol insoluble. Total S	10	17
Ethanol soluble. Total S	31	83
Ethanol soluble. Free sulphate	148	6
Ethanol soluble. Ethereal sulphate	122	0
Ethanol soluble. Bromine-oxidizable S	12	0

Again a very slight activity was observed in the bromine-oxidizable fraction, actually lower than in the first experiment. It was proved that this activity was not due to ergothioneine which, after isolation as phosphotungstate, was entirely devoid of activity.

Experiments with [³⁵S]thiolhistidine

[³⁵S]Thiolhistidine (1 g.) giving 54×10^5 counts/min. (calculated from the counts at 'infinite thickness' using an end-window counter) was added to water (200 ml.) containing sufficient 2N-hydrochloric

acid to effect solution; 50 ml. portions were mixed with the food of boar A on 4 successive days. It should be borne in mind that the same boar had received radioactive sulphate 5 weeks previously. The distribution of radioactivity can be seen from Table 4 which shows that orally administered thiolhistidine is rapidly excreted in an unchanged state in the urine; the bromine-oxidizable fraction of the first collection having high radioactivity. By the 15th day the radioactivity of this fraction was negligible. There was no incorporation of thiolhistidine into blood plasma or cells. The radioactivity of the seminal plasma on the 1st day after cessation of feeding could have been due to urinary contamination; less than 5 ml. of urine would have accounted for the observed activity of the semen (250 ml.). On the other hand, the possibility exists that as a result of feeding thiolhistidine, some of this compound may actually have passed through the male accessory glands and have been secreted into the seminal plasma. The prompt fall in activity of the bromine-oxidizable fraction of seminal plasma shows clearly that seminal ergothioneine has not been labelled and cannot therefore be derived from thiolhistidine.

Attention may be drawn to the rise in radioactivity of the ethanol-soluble portion of the sperm shown in the collections on the 8th and 15th day. This may be taken as indicating a slow but definite incorporation of ³⁵S into the spermatozoa; this radioactivity could have been derived from the H₂³⁵SO₄ administered to the same boar in the previously described experiment.

Table 4. *Radioactivity of BaSO₄ prepared by the fractionation of the sulphur-containing constituents of boar urine, blood and semen obtained after feeding [³⁵S]thiolhistidine*

(Values are expressed as counts/min. in 1 sq.cm. sample of 'infinite thickness'.)

	Days after last dose				
	1	8	15	22	36
	Counts				
Seminal plasma					
Ethanol insoluble. Total S	19	10	17	10	5
Ethanol soluble. Total S	204	75	25	27	14
Ethanol soluble. Free + ethereal sulphate	189	115	45	36	20
Ethanol soluble. Bromine-oxidizable S	845	43	56	42	33
Sperm					
Ethanol insoluble. Total S	44	59	28	15	10
Ethanol soluble. Total S	88	504	490	23	1
Blood					
Total S	28	10	7	8	5
Plasma. Total S	36	14	4	7	3
Cells. Total S	12	7	5	6	8
Urine					
Total S	397	75	60	50	37
Free sulphate	153	92	67	62	40
Ethereal sulphate	198	102	66	56	42
Bromine-oxidizable S	8800	150	0	3	0

Table 5. *Radioactivity of BaSO₄ prepared by the fractionation of the sulphur-containing constituents of boar urine, blood and semen obtained after feeding 1 mc. [³⁵S]methionine*

(Values are expressed as counts/min. in 1 sq.cm. sample of 'infinite thickness'.)

	Days after last dose						
	4	11	18	25	32	39	53
	Counts						
Seminal plasma							
Ethanol insoluble. Total S	223	277	215	194	198	153	126
Ethanol soluble. Total S	117	154	135	138	58	—	—
Ethanol soluble. Free + ethereal sulphate	136	160	67	56	55	—	—
Ethanol soluble. Bromine-oxidizable S	180	114	103	88	60	—	—
Sperm							
Ethanol insoluble. Total S	89	74	86	179	199	139	144
Ethanol soluble. Total S	17	12	12	111	80	62	52
Blood							
Total S	366	318	235	176	160	123	91
Plasma. Total S	561	387	282	175	162	111	88
Cells. Total S	153	174	152	174	160	157	103
Urine							
Total S	441	224	119	66	67	47	39
Free sulphate	531	231	116	72	64	57	41
Ethereal sulphate	219	162	119	59	53	41	20
Bromine-oxidizable S	21	24	20	0	0	0	0

Experiments with [³⁵S]methionine

[³⁵S]Methionine (13 mg.) containing 1 mc. ³⁵S was dissolved in 200 ml. water. Portions (50 ml.) of this solution were mixed with the food of boar A on 4 successive days starting 6 weeks after the termination of the thiolhistidine feeding. The results are set out in Table 5.

It can be seen that an appreciable radioactivity is present in the bromine-oxidizable fraction of seminal plasma as early as the 4th day after cessation of feeding even though the total amount of administered methionine was comparatively small, representing only 1 mc. radioactivity as against 10 mc. of the ³⁵SO₄ used in the previously described experiment. Urinary contamination of the seminal plasma

can be excluded in view of the very low activity of the bromine-oxidizable fraction in the urine. Moreover, the level of labelling of the bromine-oxidizable fraction in seminal plasma was well maintained for several weeks. The fact that this activity is due to ergothioneine was confirmed by the actual isolation of ergothioneine from the semen.

The combined ethanol-soluble portions, containing 35 mg. ergothioneine from 380 ml. semen, collected on the 39th and 53rd day of the experiment recorded in Table 5, were treated with 4 vol. ethanol. The precipitate was discarded after centrifugation and the supernatant liquid was evaporated under reduced pressure nearly to dryness. The residue was

Fig. 1. Radioactivity of BaSO_4 prepared by fractionation of the sulphur-containing constituents of boar blood and semen obtained after feeding 1 mc. ^{35}S methionine. $\bigcirc \cdots \bigcirc$, Sperm (ethanol-insol.); $\bigcirc \cdots \bigcirc$, seminal plasma (ethanol-insol.); $\bigcirc \cdots \bigcirc$, blood cells; $\bullet \cdots \bullet$, blood plasma.

diluted to 50 ml. with water and made 0.2N with respect to H_2SO_4 . Phosphotungstic acid (10%, w/v) was added until precipitation was complete. The precipitated ergothioneine phosphotungstate was decomposed with $\text{Ba}(\text{OH})_2$ solution; after concentration and crystallization from ethanol, 17.4 mg. ergothioneine were obtained. Although this ergothioneine preparation did not give a ninhydrin reaction, it was further purified by partition chromatography using Whatman no. 1 paper and butanol/acetic acid/water (50 : 10 : 40, v/v) mixture. The position of the ergothioneine was detected by spraying three thin strips cut from the edges and middle of each square, with a 0.4% (w/v) ethanolic solution of 2:6-dichloroquinone chloroimide. The

ergothioneine-containing portions of the chromatograms were cut out and eluted with hot water.

The ergothioneine thus isolated gave a count of 37/min./sq.cm. at 'infinite thickness'. The absence of any ninhydrin-reacting material indicates freedom from amino-acids, and the method of isolation would have eliminated sulphates, sulphides, sulphites, thiocyanates and thiosulphates as possible impurities. It can therefore be concluded that sulphur fed as methionine is incorporated into seminal ergothioneine.

It can also be seen from Table 5 that sperm, and in particular, the ethanol-insoluble portion, acquire an appreciable radioactivity. This, however, reaches the maximum only 4-5 weeks after the administration of ^{35}S methionine. With regard to blood it is interesting to note the high initial labelling of the blood plasma (561) in contrast to the blood cells (153). By the 4th week, however, the activity was the same in cells and plasma (Fig. 1).

Experiments with ^{35}S ergothioneine

^{35}S Ergothioneine (0.4 g.) giving 2×10^7 counts/min. (calculated from the count at 'infinite thickness' using an end-window counter) was dissolved in 400 ml. water; 30 ml. of this solution was mixed with the food of boar A on 13 successive days, starting 4 months after the termination of the methionine experiment, by which time the activity of the semen due to methionine had reached a very low level.

Table 6. Radioactivity of BaSO_4 prepared by the fractionation of the constituents of boar urine, blood and semen collected on the 5th and 9th days after feeding ^{35}S ergothioneine

(Values are expressed as counts/min. in 1 sq.cm. sample of 'infinite thickness'.)

Seminal plasma (ethanol soluble)	
Free + ethereal sulphate	79
Bromine-oxidizable S	695
Blood	
Plasma. Total S	6
Cells. Total S	32
Urine	
Total S	4
Free sulphate	2
Ethereal sulphate	2
Bromine-oxidizable S	2

Urine samples were collected on the 3rd and 5th days after feeding had been terminated, and semen was collected on the 5th and 9th days. The combined ejaculates represented 1 l. of semen and contained 124 mg. of ergothioneine. The bromine-oxidizable fraction from the seminal plasma was radioactive (Table 6). As both the urine samples had negligible

activity this could not have been due to urinary contamination. It is evident, therefore, that orally administered ergothioneine is incorporated into seminal plasma by the boar.

Examination of the blood showed higher labelling in the cells than in the plasma.

DISCUSSION

The demonstration that inorganic sulphate is unable to act as a source of sulphur for either blood or seminal ergothioneine is in accordance with observations on the metabolic inertness of sulphate in general. Sulphate is not utilized readily in the animal body except in certain cases such as in the formation of chondroitin sulphuric acid and ethereal sulphates (Dziewiatkowski, 1949). In this respect, animals with one stomach such as the pig, behave differently from ruminants. Because of the synthetic ability of the rumen micro-organisms, postulated by Rimington & Bekker (1932), ruminants are capable, for example, of efficiently utilizing inorganic sulphate for the formation of cystine and methionine (Block, Stekol & Loosli, 1951). Thiolhistidine, the corresponding amino-acid to the betaine ergothioneine, is shown by the present study to be completely inactive as a precursor of either blood or seminal ergothioneine in the pig. In this respect the pig does not differ from the rat where thiolhistidine was found by Heath (1953) to be equally inactive. These results, together with the previous observation of Mann & Leone (1953) on the complete absence of thiolhistidine, either free or bound, from both boar vesicular secretion and seminal-vesicle tissue, makes it highly improbable that thiolhistidine is involved in the normal biosynthesis of ergothioneine. Thus, the present study shows once again that the products of normal animal metabolism are not necessarily formed from chemically closely related substances.

Methionine has been shown to be capable of supplying the sulphur for the biosynthesis of ergothioneine. Our experiments establish for the first time that ergothioneine can be synthesized by an animal. Labelled ergothioneine, administered by

mouth, appeared in the semen. Hence, absorption of ergothioneine from the alimentary tract and its passage in an unchanged state into the seminal plasma is possible.

It is of interest that the spermatozoa can acquire sulphur from labelled sulphate and methionine even though the maximum radioactivity appears in the ejaculated spermatozoa only some weeks after feeding. This slow rate, however, is not altogether unexpected in view of the long time required for the processes of spermatogenesis, sperm maturation and sperm transport as compared with the time required for the formation of seminal plasma in the male accessory glands. Both [^{35}S]sulphate and [^{35}S]thiolhistidine, when fed to the pig, are rapidly excreted in the urine, mostly in an unchanged state. Heath (1953), in experiments with rats, has already shown that [^{35}S]ergothioneine is excreted in the urine partly unchanged and partly as sulphate. The urinary excretion of ^{35}S after feeding methionine was prolonged, the greater part appearing as sulphate.

There is no evidence that either labelled sulphate or thiolhistidine can provide a source of sulphur for ergothioneine in blood. Feeding [^{35}S]ergothioneine raised the activity of the total sulphur in the blood cells, thus, as in the case of the rat (Heath, 1953), dietary ergothioneine is incorporated into the cellular fraction of pig blood.

SUMMARY

1. Boars have been fed with ^{35}S -labelled sulphate thiolhistidine, methionine and ergothioneine.
2. The distribution of ^{35}S has been studied in the blood, urine and semen.
3. It has been shown that neither sulphate nor thiolhistidine is utilized in the biosynthesis of ergothioneine.
4. The sulphur of methionine is incorporated into ergothioneine.
5. Dietary ergothioneine appears in the seminal plasma of the boar.

The authors wish to acknowledge the technical assistance of Miss B. Tooth and a grant from the Agricultural Research Council for the purchase of isotopes.

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