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THE PRESENCE OF ERGOTHIONEINE IN THE CENTRAL NERVOUS SYSTEM AND ITS PROBABLE IDENTITY WITH THE CEREBELLAR FACTOR

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SUMMARY

1. Ergothioneine has been detected and quantitatively estimated in the brains of mice, rats, guinea-pigs, rabbits, cats and sheep. It is present in the cerebellum in amounts ranging from about 3·0 μg/g in the cat to 10 μg/g in the guinea-pig. Amounts in the cerebral hemispheres are much smaller, ranging from $<$ 0·1 μg/g in the cat to 1·6 μg/g in the guinea-pig.

2. Large amounts of ergothioneine (about 30 μg/g) were found in the optic nerves of the rabbit. None was detected in the dorsal columns of the spinal cord of the same species.

3. Ergothioneine has an excitatory action on the electrical activity of the cerebellum of the decerebrate rabbit and the excitatory actions of cerebellar extracts on this preparation are completely accounted for by their contained ergothioneine.

4. It is concluded that the cerebellar factor of Crossland & Mitchell (1956) is identical with ergothioneine.

INTRODUCTION

In 1956, Crossland & Mitchell reported that extracts of cerebellum, suitably injected into the cerebral circulation of the decerebrate rabbit, produced an increase in the electrical activity of the cerebellum. The material responsible for this effect was provisionally named the cerebellar factor; it could not be identified with any substance then known to occur in the brain. Later experiments demonstrated that the cerebellar factor, though occurring in larger amounts in the cerebellum than in other large areas of the brain, was also present in some localized regions of the central nervous system, particularly the optic nerves (Crossland, 1957; Crossland, Garven & Mitchell, 1959). The optic nerves, like the cerebellum, contain little acetylcholine and it seemed possible that the cerebellar factor might be particularly involved in the activity of non-cholinergic neurones.

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The cerebellar factor has little or no activity on any of the conventional pharmacological preparations so far tested and the method used for its detection does not form a suitable basis for repeated quantitative assays. These circumstances have prevented the isolation and rigorous chemical identification of the active material but a number of its properties have been determined. In this paper it is shown that these properties, some of which have already been briefly reported (Crossland *et al.* 1959) parallel those of ergothioneine. It is also shown that ergothioneine is present in brain, particularly in the cerebellum and optic nerves, and that ergothioneine has an action, similar to that of the cerebellar factor itself, on the electrical activity of the cerebellum.

A preliminary account of this work has already been published (Crossland, Woodruff & Mitchell, 1964).

METHODS

Throughout this paper, the designation 'cerebellar factor' is restricted to the acid-labile material present in extracts of nervous tissue and capable of augmenting the electrical activity of the cerebellum.

Detection of the cerebellar factor

The presence or otherwise of the cerebellar factor was determined by the intra-carotid injection of test solutions into the decerebrate rabbit and recording the effect produced on the electrical activity of the cerebellum. The method was essentially the same as that described by Crossland & Mitchell (1956) except that, in some experiments, the Ediswan pen-writing oscillograph was replaced by a 'Mingograf' ink-writer (Elema, Stockholm) whose writing device has a frequency response of up to 900 c/s and which is, therefore, more appropriate for recording fast cerebellar activity. Roughly quantitative assays of the cerebellar factor were made by comparing the minimum volume of test solution required to produce cerebellar excitation with the corresponding volume of an aqueous extract of a stock acetone powder of sheep cerebellum.

Solubility and stability of the cerebellar factor

An acetone-dried powder of sheep or rabbit cerebellum was used to provide a stable and concentrated source of the cerebellar factor, which was extracted from the powder by boiling with 6 times its weight of water (or an appropriate saline solution) for 2–3 min and centrifuging off the insoluble residue. Sheep cerebellum was obtained as soon as possible, and always within 1 hr, after the animal's slaughter. Samples of visual cortex (which contains no cerebellar factor) were taken at the same time, to provide control powders. The tissue, removed in the cold room of the abattoir, was packed in ice in vacuum flasks and the powder was prepared immediately on return to the laboratory.

Rabbits were anaesthetized with pentobarbitone, bled out and the powders made immediately.

The powders were made by homogenizing the tissue in a fivefold excess of dry, chilled acetone which was changed at least six times during the homogenization. The final homogenate was filtered, the residue washed with dry acetone followed by ether and allowed to dry, with constant grinding, at room temperature. The dry powders were stored in an evacuated desiccator over phosphorus pentoxide.

Solubility. When determining the solubility of the cerebellar factor in solvents immiscible with water, an aqueous extract of acetone powder was shaken with the appropriate solvent and the solvent phase removed. It was evaporated to dryness at 40° C *in vacuo* and the

residue taken up in 2 ml. sodium chloride solution (0·9 g/100 ml.). The saline solution and the treated extract (after removal of traces of dissolved solvent) were tested for activity. With solvents miscible with water, the powder was boiled in 100 ml. solvents/g, the suspension centrifuged, the supernatant solution evaporated to dryness and taken up in saline, for testing as before.

Stability. Hydrochloric acid or sodium hydroxide was added to 1 ml. portions of a saline extract of acetone powder to give a final pH ranging from 1 to 14. Some samples were left at room temperature (20° C) for periods of up to 24 hr, others were placed in a water-bath at 100° C for varying periods between 5 sec and 10 min. They were then cooled, neutralized and tested.

Paper chromatography of brain extracts

Chromatography of cerebellar extracts. One millilitre portions of an acid-alcohol extract of fresh sheep cerebellum, each sample containing the extract from 1·2 g of tissue, were applied in 2 cm bands to Whatman no. 1 chromatography paper. The extracts were examined by one-dimensional chromatography in three solvent systems: butanol–acetic acid–water (4:1:5 by vol., upper phase used), phenol–water and collidine–water. The last-named solvent mixture was run in the descending direction; ascending chromatography was employed for the other two mixtures. Development proceeded for approx. 15 hr and the papers were then washed in ether and dried in air. They were cut into horizontal strips which were eluted with 2–3 ml. water for 12 hr. Residual solvent was removed from the eluates by shaking with several small portions of ether which was itself removed in a stream of air. The several eluates were tested for the presence of a cerebellar factor whose approximate R_F value was determined by reference to the source of those eluates in which it was detected.

Detection of ergothioneine. Evidence for the presence of ergothioneine was sought both in crude brain extracts and in fractions collected in the course of the column-chromatographic procedure described below.

The extracts used were trichloroacetic-acid extracts of rabbit and cat cerebellum or aqueous extracts of acetone powders of sheep cerebellum. Similar extracts of cerebral hemispheres (rabbit and cat) or visual cortex (sheep) were run as controls. The solutions were washed with ether, evaporated to dryness *in vacuo* at 40° C and taken up in minimal quantities of 60% (v/v) ethyl alcohol for application to the chromatography paper. One-dimensional descending chromatograms were run in three solvent systems: ethanol–water (78:22 by vol.); butanol–acetic acid–water (4:1:5 by vol.) and phenol–water. After 14–20 hr development, the chromatograms were sprayed with diazotized sulphanilic acid or with 2,6-dichloroquinone-*p*-chloroimide (DCQ) reagent. In other experiments, a two-solvent system was used: extracts, each of about 1 g of tissue, were run in ethanol–water and a narrow strip of the developed chromatogram was sprayed with diazotized sulphanilic acid. This located activity in the region R_F 0·55–0·65 and the stained strip was used as a guide to the position of this material in the rest of the chromatogram. The appropriate area of the paper was eluted with 10 ml. of solvent, the eluate concentrated to a small volume and subjected to one-dimensional chromatography in butanol–acetic acid–water or phenol–water mixtures.

Alcohol extracts of acetone powder of sheep cerebellum were also run on thin layers of silica gel, using the same solvent systems and locating agents as were employed in paper chromatography.

Eluates from the alumina columns, evaporated to a small volume, were subjected to one-dimensional paper chromatography in butanol–acetic acid–water, ethanol–water and propanol–acetic acid–water (75:1·5:23·5 by vol.) solvent mixtures.

Quantitative determination of ergothioneine

A method essentially the same as that developed by Melville and his colleagues (Melville & Horner, 1953; Melville, Horner & Lubschez, 1954) was used for the determination of ergothioniene. The tissue was homogenized with trichloroacetic acid (10 g/100 ml.) using

2 ml. acid/g of tissue. The insoluble material was centrifuged off and was washed with 1 vol. diluted trichloroacetic acid (5 g/100 ml.). The combined supernatants followed by water were passed through a column of Amberlite IRA 410 in the acetate form and the eluates were evaporated to dryness *in vacuo* at 40–45° C. They were taken up in 2 ml. of a formic acid–ethanol–water mixture (1 g formic acid in a mixture of 75 ml. ethanol and 25 ml. water) and adsorbed on to an alumina column through which the formic acid–ethanol–water mixture was then passed at a rate of about 10 ml./hr. The eluates were collected in 1–2 ml. portions in an automatic fraction collector. The individual fractions were evaporated to dryness in air at 40–45° C and redissolved in 3 ml. carbonate–citrate buffer. Each solution was cooled to 0° C and 0·5 ml. diazotized sulphanilic acid was added. Forty-five seconds later the mixture was removed from the ice-bath and to it was added 10N sodium hydroxide. Under these conditions ergothioneine gives, within a few minutes, a characteristic magenta colour (the Hunter reaction). The ergothioneine content of eluates showing a positive Hunter reaction was determined from their optical density at 540 mμ. The minimum quantity of ergothioneine assayable by this method is about 0·5 μg.

The Hunter reaction was also used to detect the presence of ergothioneine in qualitative tests.

RESULTS

Comparison of the properties of ergothioneine and the cerebellar factor

The cerebellar factor was found to be more stable in alkaline than in acid solution. No activity was detected when cerebellar extracts at pH 1 were kept at 100° C for 2 min but in alkaline solution (pH 14) boiling had to be continued for 10 min before activity disappeared. At room temperature (20° C) no loss of activity occurred in 24 hr. The stability of ergothioneine in acid solution is very much reduced in the presence of oxidizing agents: when added to brain extracts (5 μg ergothioneine in a trichloroacetic acid extract of 500 mg rabbit brain) its stability showed the same pH and temperature dependence as did the cerebellar factor. Ergothioneine and the cerebellar factor had similar solubilities in the solvents tested, both being soluble in ethanol and *n*-butanol and insoluble in ether, chloroform and benzene. In Table 1, the chromatographic characteristics of the cerebellar factor, as far as they could be determined in relatively crude tissue extracts within the limitations of the original assay method, are compared with those for ergothioneine as quoted in the literature or obtained in the course of the present experiments.

Presence of ergothioneine in the central nervous system

It proved impossible to detect ergothioneine in brain extracts by one-dimensional paper chromatography. Other material was present in the position at which ergothioneine should have been found and this prevented the satisfactory demonstration of the characteristic colour reactions between ergothioneine and the locating reagents used. Authentic ergothioneine, added in 10 μg quantities to brain extracts, also remained undetected in the one-dimensional systems. Material behaving like ergothioneine was, however, readily demonstrated in extracts of cat and sheep

cerebellum using the two-solvent system described and in sheep cerebellum, by one-dimensional, thin-layer chromatography. The results of typical experiments using these methods are shown in Table 2.

The application of Melville's column-chromatographic method (Melville & Horner, 1953; Melville *et al.* 1954) provided convincing confirmatory evidence of the presence of ergothioneine in brain extracts and permitted its quantitative determination. Elution of material, adsorbed on to the alumina column from brain extracts, yielded several fractions which reacted with diazotized sulphanilic acid but, on subsequent treatment with alkali, only one group of fractions produced the magenta colour of the

TABLE 1. Comparison of the chromatographic properties of the cerebellar factor and ergothioneine

R_F values	Cerebellar factor	Ergothioneine
Butanol–acetic acid–water	0·24–0·36	0·20–0·28 (12 determinations, mean 0·25) 0·25*
Collidine–water	0·4–0·5	0·32†
Phenol–water	0·7–0·8	0·82–0·90 (5 determinations, mean 0·86) 0·87† 0·95*

* Wolf (1962). † Heath, Lawson & Rimington (1951).

TABLE 2. Chromatographic evidence of the presence of ergothioneine in brain extracts

Paper chromatography

Extracts were first run in ethanol–water; material in the region R_F 0·55–0·65, reacting with diazotized sulphanilic acid, was eluted and subjected to one-dimensional chromatography in the mixtures indicated

	R_F of ergothioneine-like spot	
Material	Butanol–acetic acid–water	Phenol–water
Ergothioneine	0·22	0·87
Sheep cerebellum	0·22	0·85
Cat cerebellum	0·28	0·80
Thin-layer chromatography		
Ergothioneine	0·01	0·45
Sheep cerebellum	0·01	0·43

These spots all gave the same orange colour with both diazotized sulphanilic acid and the DCQ reagent. In the cat cerebellum extract, other spots reacting with DCQ were found. In butanol–acetic acid–water, they were at R_F 0·27 and 0·38, giving blue and red-brown colours respectively; in phenol–water a blue colour was given at R_F 0·35.

Hunter reaction, accompanied by the characteristic odour of trimethylamine. Ergothioneine, run concurrently on another column, appeared in a similar fraction of the eluate. Thus, in the experiment illustrated in Fig. 1, both ergothioneine and the material from brain giving the Hunter reaction appeared in the 25–37 ml. portions of the respective eluates. The Hunter reaction was not obtained if the eluted material was first boiled for 2 min in acid, nor was any reacting eluate produced if the brain extract

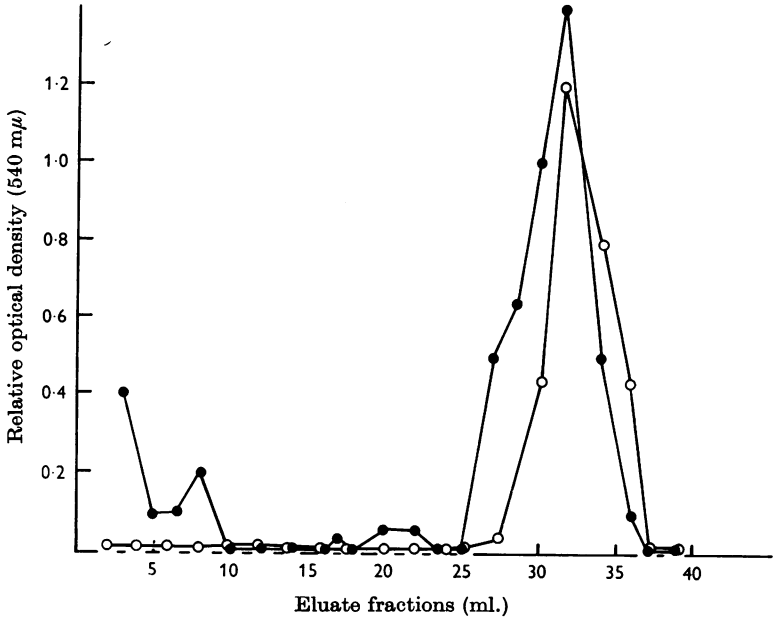


Fig. 1. Elution of ergothioneine (10 μg, open circles) from an alumina column and of an extract of guinea-pig cerebellum (1·2 g, closed circles) run concurrently on a second column. Abscissa: eluate fractions in ml. Ordinate: relative optical density at 540 mμ after treatment with diazotized sulphanilic acid and 10N sodium hydroxide.

itself had been similarly treated with acid and neutralized before application to the column. Eluates giving the Hunter reaction were subjected to one-dimensional paper chromatography in two different solvent systems. Their chromatographic behaviour was indistinguishable from that of ergothioneine (Table 3).

Other than ergothioneine, the only naturally occurring substance giving the Hunter reaction is thiolurocanic acid (Lawson, Morley & Woolf, 1951; Yanosugonolha & Appleman, 1957). This compound has, as yet, not been found in mammalian tissues and, though it gives the same colour as does ergothioneine with alkalinized sulphanilic acid, it does so without the

liberation of trimethylamine. Further, ergothioneine and thiolurocanic acid could be readily distinguished from one another on paper chromatography (Table 3) and in an experiment when both were eluted from a mixture applied to the alumina column, ergothioneine appeared in the 25–35 ml. fractions and thiolurocanic acid in the 45–50 ml. fractions.

TABLE 3. Chromatographic evidence of the identity of ergothioneine and the 'ergothioneine-fraction' eluted from the alumina column

(Eluates and authentic substances subjected to one-dimensional paper chromatography in solvents named.)

	R_F values				
	Butanol–acetic acid–water	Ethanol–water	Propanol–water	Ninhydrin reaction	Colour with diazotized sulphanilic acid
Ergothioneine fraction (25–35 ml.)	0·24	0·58	.	–	Orange
Ergothioneine	0·22	0·58	0·38	–	Orange
Thiolurocanic acid	0·78	.	0·60	–	Red-brown
Histidine	0·08	.	0·16	+	Crimson
Fraction 100–110 ml.	0·08	.	0·15	+	Crimson

Quantitative determination of ergothioneine in brain extracts

The alumina column permits a complete separation of ergothioneine from amino acids, many of which react with diazotized sulphanilic acid to give coloured products which, if present, would obscure the magenta colour of the Hunter reaction. The completeness of the separation is illustrated by the experiment summarized in Table 3: the first reacting amino acid to appear in a sufficient quantity to be detected chromatographically was histidine. Ergothioneine was cleared from the column in the first 35 ml. of eluate; histidine was not detected until another 65 ml. of eluant had passed through.

In the absence of interfering material, ergothioneine can be determined in the active eluates by simple colorimetry. Loss of ergothioneine on the column is relatively small: in six experiments, amounts of ergothioneine ranging from 5–200 μg were taken through the same extraction process as was applied to brain tissue and were recovered in an average yield of 92 %.

The method was applied to the determination of the amounts of ergothioneine in extracts of the cerebellum and cerebral hemispheres of the rabbit, rat, guinea-pig, mouse, cat and sheep. In all six species the amount of ergothioneine in the cerebellum was several times greater than in the hemispheres (Table 4). In the rabbit a comparison was also made of the ergothioneine content of the optic nerves and the dorsal columns of the

spinal cord. Table 5 presents the results of three such comparisons: the optic nerves were found to contain some 30 times as much ergothioneine as the dorsal columns and about 6 times as much as the cerebellum of the same species.

TABLE 4. Ergothioneine content (μg/g fresh tissue) of the cerebellum and cerebral hemispheres of various mammalian species

	Cerebellum	Cerebral hemispheres
Sheep	3·0 (100)	0·6 (100)
Cat	3·0, 3·3 } Mean 3·2	0·0, 0·1 } Mean < 0·1
Rat	3·0 (5), 3·0 (5), 7·5 (10), 3·2 (5) } Mean 4·8	0·5 (5), 0·0, 0·8 (10), 0·2 (5) } Mean 0·6
Rabbit	4·3 (3), 4·6, 5·0, 5·5 (2), 5·8 (2) } Mean 5·0	0·9, 1·2, 1·2, 1·3, 1·5 } Mean 1·2
Mouse	5·0 (28)	0·0 (10)
Guinea-pig	12·0 (4), 9·9 (3), 7·6 (3) } Mean 10·1	1·5 (4), 1·8, 1·6 (3) } Mean 1·6

When determinations were made on extracts of pooled brains the number pooled is indicated in parentheses.

The value for the sheep was obtained from assay of a sample of a large batch of acetone powder on the assumption that 1 g powder is equivalent to 5 g fresh tissue.

Fresh brain was used in all the other determinations.

TABLE 5. Ergothioneine content (μg/g) of the optic nerves and dorsal columns of the spinal cord of the rabbit

Optic nerves	Dorsal columns
21	1·0
36	1·0
29	0·8

Effect of ergothioneine on the electrical activity of the cerebellum

Ergothioneine caused an increase in the electrical activity of the cerebellum of the decerebrate rabbit (Fig. 2). The time interval between injection and the electrical change was similar to those previously reported for the cerebellar factor. In seven experiments the mean latency of the response to ergothioneine was $9{\cdot}2 \pm$ S.E. 1·5 sec; for the cerebellar factor and histamine the reported latencies were $13{\cdot}6 \pm 2{\cdot}4$ (38) and $61{\cdot}5 \pm 4{\cdot}1$ (26) sec respectively (Crossland & Mitchell, 1956).

The sensitivity of the decerebrate rabbit preparation varied considerably from animal to animal. In some, the cerebellum would not respond to doses of ergothioneine smaller than 2 μg, but the most sensitive preparations regularly reacted to the administration of 0·1 μg. In the individual

experiment, the threshold dose of acetylcholine was always about one-third that of ergothioneine, as low as 0·03–0·04 μg in the most sensitive preparations.

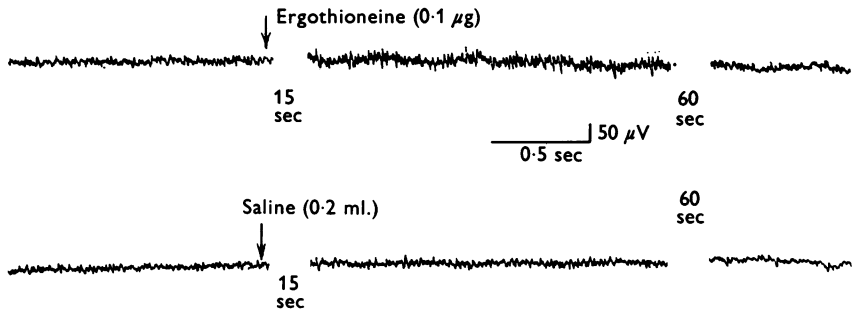
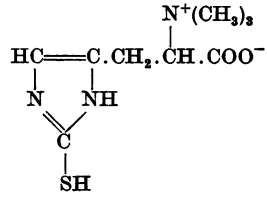


Fig. 2. Pen-oscillograph record of the electrical activity of the cerebellum of the decerebrate rabbit, before and after the injection of ergothioneine (0·1 μg in 0·2 ml. saline; upper record) and of 0·2 ml. saline (lower record).

In these recent experiments, the magnitude of the changes in the cerebellar record following the injection of active substances was often much smaller than that observed in the work previously reported. The cause of this reduced response has not yet been satisfactorily explained but it was shown equally towards acetylcholine, histamine, the cerebellar factor and active extracts of other areas of the brain, whose relative effectiveness has remained unaltered. The cerebellar response is all-or-none in character, supra-threshold amounts of active extracts causing little or no increase in the size of the response over that evoked by threshold doses.

DISCUSSION

A characteristic feature of the cerebellar factor, which enables it to be distinguished from other pharmacologically active substances likely to be present in brain extracts, is its stability on boiling in alkali for periods of up to 10 min and its ready inactivation on boiling in solutions more acid than pH 5·5. A search in the chemical literature revealed that, among naturally occurring compounds, ergothioneine



possesses these properties, its relative instability in acid arising from the ease of oxidation of the sulphydryl group which is, however, extremely resistant to alkali. Under alkaline conditions fission eventually occurs elsewhere in the molecule with the formation of thiolurocanic acid and the liberation of trimethylamine.

The other properties of the cerebellar factor, so far as they were known, appeared to be not dissimilar to those of ergothioneine. In particular the similar R_F values of ergothioneine and the cerebellar factor (determined on impure extracts and by a technique that did not allow its precise localization), in three separate solvent systems, suggested a relation between the two substances (Table 1). The structural resemblances between ergothioneine (the betaine of thiolhistidine) and histamine, which shares with acetylcholine and the cerebellar factor the property of increasing the electrical activity of the cerebellum, provided additional reasons for examining brain extracts for the presence of ergothioneine.

The results presented in this paper provide good evidence that ergothioneine is indeed present in the brain. Material indistinguishable from ergothioneine was detected by paper chromatography of brain extracts in a two-solvent system and by column chromatography, using an established method for its detection and determination in tissue extracts. The ergothioneine-containing eluates from the alumina columns were contaminated neither with the one known naturally occurring substance (thiolurocanic acid) which would have given the same colour reaction as ergothioneine nor with amino acids which would have invalidated the quantitative assay by modifying the colour of the Hunter reaction by their own reaction with diazotized sulphanilic acid.

In the only earlier reference to the subject, Exner & Kartchner (quoted by Melville, 1959), reported that ergothioneine was not present in the brain. They too used Melville's method but they examined only the rat and it is not clear whether the extract they used was made from the whole brain of the rat or only from the cerebral hemispheres, whose ergothioneine content is very low. In the rat, the mass of the cerebellum is about one-sixth that of the hemispheres and reference to Table 4 indicates that even the inclusion of the cerebellum would increase the over-all brain ergothioneine level to no more than 0·70 μg/g, an amount which might well have passed undetected in a study in which the other rat tissues examined had ergothioneine levels in the range of 4–133 μg/g.

In all six species investigated, ergothioneine was found in larger amounts in the cerebellum than in the rest of the brain; of the two individual tracts examined in the rabbit, the optic nerve contained large amounts of ergothioneine, the dorsal columns of the cord had none. This distribution corresponds, qualitatively at least, with that already reported for the cerebellar factor (Crossland *et al.* 1959), though the amounts found in the optic nerves are surprising since the amount of cerebellar factor detected in these nerves was usually no higher than in the cerebellum itself. An exception was provided by the horse, whose optic nerves yielded rather more cerebellar factor per gram than did the cerebellum (Crossland, 1957).

On the electrical activity of the cerebellum, ergothioneine has an action similar in form, latency and duration to that evoked by the cerebellar factor. Of the other substances so far tested, only acetylcholine and histamine have been found to have a cerebellar excitatory action in very small doses and the action of histamine is delayed for some time after injection. In our most sensitive preparations an obvious cerebellar response was observed with as little as 0·1 μg of ergothioneine. This amount of ergothioneine would be present in about 20 mg of rabbit cerebellum—the minimum dose of cerebellar extract which stimulated cerebellar activity in the experiments of Crossland & Mitchell (1956). In the rabbit, the ergothioneine content of the cerebellum is about three times the acetylcholine content of other areas of the brain and in our active preparations the threshold dose of ergothioneine was approximately three times that of acetylcholine. These facts are consistent with the finding (Crossland & Mitchell, 1956) that, on the electrical activity of the cerebellum, extracts of rabbit cerebellum (whose activity was due to the cerebellar factor) were equipotent, weight for weight, with extracts of cerebral hemispheres whose action was due to acetylcholine.

The cerebellar factor was originally characterized as an acid-labile compound, present in the cerebellum in higher concentration than in the rest of the brain and having an excitatory action on the cerebellum. Ergothioneine possesses these characteristics and its other properties are similar to those subsequently described for the cerebellar factor. Since ergothioneine is present in the cerebellum in sufficient quantity to account for the whole of the cerebellar excitatory action of cerebellar extracts, the conclusion is inescapable that it is identical with the cerebellar factor, though the discrepancy between the ergothioneine and the cerebellar factor content of the optic nerves remains unexplained.

Ergothioneine was isolated from ergot by Tanret in the early years of the century (Tanret, 1909) but, although it is widely distributed in plant and animal tissues, no definite biological function has yet been ascribed to it. Much of the available evidence suggests that, in those mammalian tissues which have hitherto been studied, ergothioneine is of exclusively dietary origin (Melville *et al.* 1954; Melville, Horner, Otken & Ludwig, 1955) but it is clearly possible that the brain may be uniquely capable of synthesizing the compound.

Since the preliminary account of this work was published (Crossland *et al.* 1964) it has been briefly reported that ergothioneine, iontophoretically applied to cells of the cerebral and cerebellar cortices, has little direct action on cerebellar neurones and none on those in the cerebral cortex (Krnjević, Randić & Straughan, 1965). It is not known how much ergothioneine actually reached the cells in these experiments but, in any event,

any physiological action that ergothioneine may have in the brain need not involve a direct action on the sub-synaptic membrane. The presence and uneven distribution of ergothioneine in the central nervous system and its action on the electrical activity of the cerebellum certainly suggest the possibility that it may exert a central regulatory function of some kind.

The authors wish to express their indebtedness to Dr P. R. Carnegie, who gave valuable assistance and advice concerning chromatographic techniques during the early stages of this investigation. The work could not have been completed without the assistance of personal grants (to J.C.) from the Government Grants Committee of the Royal Society and the Medical Research Council whose support is gratefully acknowledged.

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