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Copper Concentration and Cytochrome-Oxidase and Ribonuclease Activities in the Brains of Copper-Deficient Lambs

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The association of degenerative changes in the central nervous system of lambs in enzootic ataxia or 'swayback' with low concentrations of copper in blood and liver has been clearly established (Innes & Shearer, 1940; Bennetts & Beck, 1942). Gallagher, Judah & Rees (1956*a, b*), working with rats, and Gubler, Cartwright & Wintrobe (1957), using pigs, demonstrated that copper deficiency resulted in a marked fall of cytochrome-oxidase activity in several tissues. These later findings, coupled with the work of Wainio, Wende & Shimp (1959), which provided clear evidence that copper is an integral part of the cytochrome-oxidase molecule, prompted Howell & Davison (1959) to compare the cytochrome-oxidase activity of brain tissue from normal and swayback lambs. They demonstrated a significant reduction in cytochrome-oxidase activity in homogenates of frontal cortex, cerebellar peduncle and caudate nucleus prepared from brain tissue of lambs with ataxia. The copper content of these brain fractions from ataxic lambs was lower than in corresponding tissues from the normal lambs used in their study. The mean copper concentration of the liver of their clinically normal lambs (109 ± 13 p.p.m.) falls within the range (74–430 p.p.m.) quoted by Cunningham (1946) as being that found in young lambs reared outside copper-deficient areas. However, it has been our experience and that of other workers that many lamb flocks fail to show cases of ataxia even though the animals would be regarded as severely copper-deficient on the basis of the low copper contents of liver and blood.

This point led us to examine possible relationships between liver and brain copper and brain cytochrome-oxidase activity in swayback lambs

and in lambs from flocks of low liver copper content in which clinical signs of swayback had not appeared. This study was also extended to a number of lambs reared by ewes that had received dietary supplements of sodium sulphate and ammonium molybdate during pregnancy (a procedure that results in copper deficiency and development of typical histological lesions of swayback in the lamb; Mills & Fell, 1960; Fell, Williams & Mills, 1961). In these earlier studies the first detectable sign of degenerative change in the central nervous system was the absence of cytoplasmic RNA in the large motor neurones of the red nucleus. This absence of RNA could arise presumably from either a failure of RNA synthesis or an increase in ribonuclease activity. This latter possibility was examined in the work reported.

MATERIALS AND METHODS

Lambs suffering from 'delayed' swayback were drawn from a number of farms in north-east Scotland. Clinically normal lambs with tissues of low copper content were taken from the Duthie Stock Farm of the Rowett Research Institute; previous work had shown that many lambs from this farm have a low copper content of blood and liver. Animals were killed by bleeding after intraperitoneal administration of sodium pentobarbitone and their brains rapidly dissected out on to ice prepared from glass-distilled water. Lateral slices of the medulla between the trapezoid body and the point of entry of the hypoglossal nerve, taken to include the area of the red nucleus, were dissected from both sides of the anterior median fissure. Approximately 0.6 g. of fresh tissue was homogenized in 10 ml. of ice-cold water for cytochrome-oxidase and ribonuclease assays.

Cytochrome oxidase. Cytochrome-oxidase activity was determined manometrically by the method of Schneider &

Potter (1943). Cytochrome *c* (C. F. Boehringer und Soehne, Mannheim, Germany) and sodium ascorbate (Roche Products Ltd., London) were used without further purification, solutions of the latter being freshly prepared daily. All assays were completed within 90 min. of the death of the animal. Results are expressed as Q_{O_2} (μ l. of O_2 /mg. dry wt./hr.).

Separate experiments with rats indicated that killing by sodium pentobarbitone had no effect on the cytochrome-oxidase activity of brain homogenates.

Ribonuclease assay. The method used was essentially that of Dickman, Aroskar & Kropf (1956), with the following modifications. Brain homogenates, 0.2, 0.4, 0.6 and 0.8 ml. for each assay, were incubated with 5 mg. of RNA prepared from baker's yeast (The Distillers Co. Ltd.) by the method of Holden & Pirie (1955). The volume of each assay system was adjusted to 1.5 ml. with tris-HCl buffer (0.05M; pH 7.5) before incubation at 37° for 1 hr. with stirring. The reaction was stopped by adding 3 ml. of the acid-ethanol reagent of Roth & Milstein (1952). The precipitate was removed by centrifuging and 1 ml. of supernatant was diluted with 3 ml. of water before determination of $E_{260\text{m}\mu}$. Appropriate blanks were prepared by adding substrate after the acid-ethanol reagent to tubes incubated with homogenate and buffer only. In this system 1 μ mg. of crystalline bovine pancreatic ribonuclease (C. F. Boehringer und Soehne) produced E 0.15 and the calibration curve was linear up to 3 μ mg. For convenience our results are expressed in terms of arbitrary units, each unit being that activity which produced an increase in E of 0.05 during the period of assay.

Copper analyses. The copper content of brain and liver tissues was determined by the method of Eden & Green (1940). Insufficient material was available from the small area of the red nucleus, after removal of samples for assays of cytochrome oxidase and ribonuclease, to permit copper determination. The copper content of the whole of the remaining brain tissue was accordingly determined after powdering the freeze-dried material.

RESULTS

Cytochrome-oxidase activity. Results are presented in Fig. 1. The mean cytochrome-oxidase activity found in the area of the red nucleus of clinically normal lambs of 1-3 months of age was Q_{O_2} 95 ± 3.1 (range 74-126 in 23 observations). The activity in normal lambs killed within 24 hr. of birth was higher but more variable between individual lambs (120 ± 11.1 ; range 74-160 in eight observations). In brain tissue from swayback lambs the cytochrome-oxidase activity was lower, with a mean Q_{O_2} 59 ± 4.5 (range 31-79 in ten observations), a finding that confirms the work of Howell & Davison (1959).

Ribonuclease activity. The ribonuclease activity of tissue taken from the area of the red nucleus appeared to be the same in both normal and swayback lambs. The activity in homogenates of normal brain tissue was 13.9 ± 1.1 units/mg. dry wt. of tissue/hr. (fourteen observations) and in swayback-

brain tissue 13.9 ± 2.0 units/mg. dry wt. of tissue/hr. (six observations). Values were similar in both 1-day-old and 1-3-month-old animals.

Copper in the liver and brain of normal and ataxic lambs. Of the thirty-one clinically normal animals whose tissues were assayed for either cytochrome-oxidase or ribonuclease activity only three had copper contents in the liver within the range for 'normal' lambs (74-430 p.p.m. quoted by Cunningham, 1946), and only seven were above Cunningham's upper limit of 34 p.p.m. for copper-deficient lambs (see Fig. 2). The mean copper content of the livers of our normal lambs was 22.5 ± 5.1 (range 3-129) μ g./g. dry wt. of tissue and of lambs showing ataxia was 12.4 ± 1.1 (range 2-64) μ g./g. dry wt. of tissue.

Thus despite there being a lower mean liver copper concentration in the livers of swayback lambs it was not possible in most instances to distinguish between clinically normal and swayback lambs on this basis. However, normal and swayback lambs could be clearly distinguished on the basis of the copper content of the whole brain. Normal lambs had a mean brain copper concentration of 7.1 ± 0.4 (range 2.7-13.3) μ g./g. dry wt. of tissue and swayback lambs 2.4 ± 0.1 (range 1.6-2.6) μ g./g. dry wt. of tissue. There appeared to be no correlation between cytochrome-oxidase activity and brain copper content when the copper content was 2.8 μ g./g. dry wt. of tissue or higher. Copper

Fig. 1. Cytochrome-oxidase activity (Q_{O_2}) in the area of the red nucleus in the lamb brain and brain copper concentration. O, Tissue from clinically normal lambs 1-3 months of age; Δ , clinically normal lambs 24 hr. old; $\bullet$, swayback lambs 1-3 months of age.

Fig. 2. Relationships between the copper contents of liver and brain of the lamb. O, Clinically normal lambs; ●, swayback lambs.

concentrations below $2.4 \mu\text{g./g. dry wt. of tissue}$ were associated with a low cytochrome-oxidase activity.

DISCUSSION

Although the association between enzootic ataxia or swayback in lambs and relatively low copper contents of the liver and blood of the lamb and its parent ewe is well established it has become increasingly clear that the low copper contents of these tissues do not invariably lead to degeneration of nervous tissue. In some areas, commonly accepted criteria of normality for blood and liver copper contents have to be treated with considerable reserve since large numbers of animals showing no clinical abnormality are severely deficient in copper as judged by these standards (e.g. Robertson, 1957).

There appears to be a much closer relationship between the ataxia resulting from degenerative changes in nervous tissue and the copper content of the brain, as was originally suggested in the work of Howell & Davison (1959). In our work not one animal out of 18 showing ataxia had a brain copper content as high as $3 \mu\text{g./g. dry wt. of tissue}$, and of

the 37 clinically normal animals examined only 2 had brain copper contents below this figure. Like Howell & Davison (1959), we cannot reconcile our findings for brain copper concentration with those of Shearer & McDougall (1944), who found no difference in the copper content of normal and swayback brain tissue. However, E. I. McDougall (personal communication) has pointed out that all their cases of swayback were neonatal and in some instances the pregnant ewes had received copper supplements during the terminal stages of pregnancy, whereas our cases and those of Howell & Davison (1959) were all delayed.

From Fig. 2 it seems probable that some cases of ataxia cannot be explained on the basis of a uniform depletion of the copper reserves of both brain and liver. Possibly, in many cases of 'delayed' swayback the normal relationships between copper in brain and copper in liver have become subject to interference in that transfer of copper to the brain has been restricted either during foetal development or during the early postnatal growth. The nature of this postulated interfering agent remains in doubt at this stage, but such interference has already been demonstrated in tissues other than brain for molybdate and sulphate influencing copper transport into erythrocytes (Dick, 1956).

In our experiments a copper concentration in the whole brain of less than $3 \mu\text{g./g. dry wt. of tissue}$ was associated in most instances with a reduced cytochrome-oxidase activity in the red nucleus. The cytochrome-oxidase activity in the area of the red nucleus in the two clinically normal animals having brain contents of 2.7 and $2.8 \mu\text{g./g. dry wt. of tissue}$ referred to in a previous paragraph showed an activity of cytochrome oxidase in the red nucleus that was near to the 'normal' mean in both instances. No continuous relationship was observed in clinically normal animals between cytochrome-oxidase activity and brain copper content. In these animals activity was quite unrelated to brain copper content, with 1-day-old lambs showing a considerably wider range of variation in activity than animals aged 1-3 months. The results plotted in Fig. 1 suggest, however, that the critical threshold concentration of copper in the brain below which deficiency begins to influence cytochrome-oxidase activity is almost identical with that below which degenerative changes and ataxia supervene, i.e. approximately $2.6 \mu\text{g./g. dry wt. of tissue}$.

Interest in possible changes in ribonuclease activity associated with changes in the copper content of brain tissue was stimulated by earlier observations of the sensitivity of this enzyme to traces of copper acting as an inhibitor *in vitro* (e.g. Kaplan & Heppel, 1956; Dickman & Aroskar, 1955). The concentrations of copper causing inhibi-

tion *in vitro* are not far removed from those present in normal brain tissue; it was postulated that copper in the brain cell might be one of the factors regulating ribonuclease activity and that deficiency of this element might lead to RNA destruction in the large motor neurones of the red nucleus at a rate that could not be made good by synthetic processes. Our results failed to support this hypothesis as no difference was found in the ribonuclease activity in this tissue taken from normal or ataxic lambs. Unless our failure to detect a change in ribonuclease activity is a reflexion of limitations inherent in the use of a homogenate technique to investigate what appears, on histological evidence, to be a local phenomenon, it must be concluded that the absence of cytoplasmic RNA from these cells is either a direct effect of copper deficiency on RNA synthesis or arises from permeability or environmental changes influencing the supply of substrates for RNA synthesis.

In this study, as in the work of Gallagher *et al.* (1956*a, b*), Gubler *et al.* (1957) and Howell & Davison (1959), a close association has been demonstrated between the activity of the copper-containing enzyme cytochrome oxidase and the manifestation of metabolic disorders consequent upon copper deficiency. Such an association may provide a useful diagnostic approach that is not subject to the difficulties inherent in the interpretation of analytical figures for copper content of tissues, particularly since, in certain circumstances, tissues can accumulate copper which is not physiologically available (e.g. Marston, 1952; Mills, 1960).

The use of cytochrome-oxidase assay as a 'screening' technique to support a diagnosis of enzootic ataxia or swayback requires far less time than either chemical determination of the copper content of tissues or histological examination of brain or spinal cord tissue. Whether or not it would be as specific as histological examination depends upon the magnitude of changes in cytochrome-oxidase activity in other disorders resulting in ataxia such as bacterial meningitis, clostridial infections, encephalomalacia and spinal abscesses. In two cases of spinal abscess examined by us, the cytochrome-oxidase activity of the area of the red nucleus was within the normal range.

SUMMARY

1. Cytochrome-oxidase and ribonuclease activities in the area of the red nucleus of the brains of swayback lambs and normal lambs of low liver copper content have been compared.

2. Cytochrome-oxidase activity was significantly lower in brain tissue from swayback lambs than in that from clinically normal lambs. No differences in ribonuclease activity were detected.

3. Clinically normal and swayback lambs could be clearly distinguished on the basis of the copper content of brain tissue. In all cases swayback was associated with brain copper concentrations of less than 3 µg./g. dry wt. of tissue. No such clear distinction was apparent on comparing liver copper concentrations of normal and affected lambs.

4. It is suggested that factors influencing the distribution of copper between the brain and other tissues may be of importance in the aetiology of swayback.

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