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THE EFFECT OF FOLATE DEFICIENCY ON NEURONAL RNA CONTENT

A QUANTITATIVE CYTOCHEMICAL STUDY

MATTI HALTIA

From the Neuropathological Laboratory, Department of Pathology I, and the Institute of Neurobiology, University of Göteborg, Göteborg and Astra Nutrition Research Laboratories, Mölndal, Sweden

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SUMMARY.—One-day-old chicks were fed a defined ration deficient in folic acid. They were killed at 4 weeks of age when they showed characteristic clinical signs of folate deficiency and extremely low whole blood folate levels. Cerebellar Purkinje cells were dissected out and their total ribonucleic acid (RNA) content was determined by Edström's microchemical technique. The total RNA content of Purkinje cells of the folate deficient chicks was significantly lower than that of control chicks fed a complete ration. The low RNA values of the folate deficient chicks were apparently not only secondary to anaemia or growth retardation, and suggest that severe folate deficiency may directly interfere with neuronal RNA synthesis. The significance of the findings is discussed with reference to human pathology.

FOLATE coenzymes play a vital role in the biogenesis of purines and pyrimidines and thus indirectly influence the synthesis of ribonucleic acid (RNA) and desoxyribonucleic acid (DNA) (*cf.* Huennekens, 1968). Consequently, the effects of folate deficiency on cellular metabolism are potentially extremely severe (Woods, 1964).

A great deal of experimental work has been done on the effects of folate deficiency (conveniently mimicked by the administration of folate antagonists) on the differentiation of the embryonal nervous system. Various types of CNS malformations have been produced by the use of folate antagonists in amphibian (Hitchings, 1955; Grant, 1960), avian (Adrian, 1958), and mammalian species (Nelson, 1960; Giroud, 1959; Newberne and O'Dell, 1961). Even human CNS malformations have been reported after the use of folate antagonists (Thiersch, 1952, 1956). Johnson (1964) studied the effects of maternal folate deficiency on cytologic phenomena in rat embryos, and found, besides a decreased mitotic rate, reduced amounts of ribosomes and histochemically demonstrable RNA in the cytoplasm of cells of the neural tube, indicating disturbances in both DNA and RNA metabolism of the developing nervous system.

No information has been published on the effect of folate deficiency on differentiated neurones. In contrast to most other cells the neurones are characterized by a high content of RNA even in the differentiated state and, in fact, have no competitors as RNA producers among other somatic cells (Hydén, 1967). Considering the vital role of folates in the biogenesis of purine bases and the effects of folate deficiency on the cells of the neural tube it appeared reasonable to assume

that folate deficiency would interfere with neuronal RNA synthesis. It was decided to test this hypothesis in view of the present lively discussion on the relationship between folate deficiency and disease of the nervous system (*cf.* Reynolds, 1968).

In the present study the effect of experimental folate deficiency on the RNA content of individual nerve cells was investigated using a quantitative cytochemical method.

MATERIALS AND METHODS

Chicks were chosen as experimental animals because nutritional folate deficiency is easily elicited in this species without the use of folate antagonists or intestinal antiseptics (Robertson, Daniel, Farmer, Norris and Heuser, 1946). Purkinje cells were used in the cytochemical work, as preliminary experiments on chicks and previous experience with other species (Jarlstedt, 1962) had shown that they constitute a uniform population with regard to their RNA content.

Experimental animals and diets.—Twelve 1 day old white Plymouth Rock chicks were fed *ad libitum* a highly purified casein–glucose diet without the addition of folic acid (modified from Campbell, McCabe, Brown and Emmett, 1945). Twelve control animals of equal starting weight were given unrestricted amounts of the same diet with folic acid added (2 mg./kg. of diet). One experimental animal died before the age of 4 weeks and was excluded from the experiment, as was a control animal of equal starting weight, so that both the experimental and the control group ultimately consisted of 11 animals.

The animals were weighed twice weekly. At the age of 4 weeks the quality of feathering was estimated using the feather score of Robertson *et al.* (1946), and blood samples were taken from the wing vein. The animals were killed by decapitation.

An additional 3 control animals were killed at the age of 3 weeks for determination of Purkinje cell RNA content. Their body weights at this age (318, 325 and 326 g.) corresponded closely to that of the smallest experimental animal (327 g.) at 4 weeks of age.

Folate determinations and haematological studies.—Folate activity in heparinized whole blood samples from the wing vein was determined microbiologically using *Lactobacillus casei* and *Streptococcus faecalis* according to Hansen (1964). Erythrocyte (RBC) counts were made and haemoglobin concentration was measured spectrophotometrically as oxyhaemoglobin as described by Hallgren (1953).

RNA determinations. The method described by Edström (1964) was used for determining the total RNA content of Purkinje cells. The cerebellum was quickly removed and fixed in Carnoy's solution for 2 hr, dehydrated, and embedded in soft paraffin. Sections were cut at 40 μ, de-paraffinized, and hydrated with 0·01 N acetic acid. Purkinje cell perikarya were dissected out from folium V of the vermis by micromanipulation and extracted with buffered ribonuclease (Worthington Biochemical Corporation, Freehold, New Jersey) solution. The extracts were evaporated to dryness on quartz slides and redissolved in a buffer solution. The amounts of RNA in the droplets were determined with a photographic-densitometric procedure. At least 40 cells, in pairs, were analysed per animal. The total number of analysed cells amounted to 1022.

RESULTS

The chicks fed the folate deficient ration became progressively lethargic after about 10 days. They mostly lay apathetically on the cage floor, but when disturbed, could adequately support themselves and did not display ataxia. Their food consumption was only slightly diminished. During the 2nd week their growth rate dropped appreciably, although the body weight of some was still within the control range at 4 weeks of age. After the 2nd week all experimental animals showed progressive retardation of feather development (Table I). One chick had a marked slipped tendon sign or so-called " perosis " (Daniel, Farmer and Norris, 1946). Cervical paralysis (Richardson, Hogan and Kempster, 1945) was not observed. The control animals remained in excellent condition.

Analysis of the blood samples (Table I) revealed that the chicks on the folate deficient diet consistently had extremely low whole blood folate values in determinations with both *L. casei* and *Str. faecalis*, as compared with the control animals. Six of the experimental animals had RBC counts, but only 2 haemoglobin concentrations below the control range ($<1{\cdot}99 \times 10^6$ RBC/mm.^{3} and $<7{\cdot}15$ g./100 ml., respectively) reflecting the development of macrocytic anemia.

The results of the microchemical determination of the total RNA content of Purkinje cells are shown in Tables II and III.

TABLE I.—*Clinical and Laboratory Criteria of Folate Deficiency. Mean Values ± S.E.M.*

	Body weight (g.)	Feather score (per cent)	Erythrocyte count (10^6/mm.^{3})	Haemoglobin concentration (g./100 ml.)	Whole blood folates (ng./ml.) *Lactobacillus casei*	Whole blood folates (ng./ml.) *Streptococcus faecalis*
Control chicks	538±18	100±0	2·47±0·09	8·0±0·2	25·8±2·4	19·6±1·5
Folate deficient chicks	405±26	23±2	1·76±0·18	7·8±0·7	3·6±1·0	1·6±0·3

TABLE II.—*Total RNA Content of Purkinje Cells*

Chick no.	Total amount of RNA (pg.) per 2 Purkinje cells Mean values ± S.E.M. Control chicks	Folate deficient chicks
1	258± 7	219± 8
2	261±11	207± 9
3	283±14	232± 6
4	269± 8	266±10
5	256±10	210± 8
6	277± 7	184±10
7	293± 9	170± 4
8	272± 7	208± 5
9	251± 7	221± 7
10	280± 8	231± 8
11	256± 9	242±10
Group mean values ±S.E.M.	269± 4	217± 8

TABLE III.—*Body Weight and Total RNA Content of Purkinje Cells of 3-week-old Control Chicks*

Chick no.	Body weight (g.)	Total amount of RNA (pg.) per 2 Purkinje cells Mean values ± S.E.M.
13	326	269± 9
14	318	252± 8
15	325	260±10
Group mean values	323	260

DISCUSSION

The three well-established criteria of folate deficiency syndrome in chicks are retarded growth, poor feathering, and macrocytic anemia (*e.g.* Campbell *et al.*, 1945; Robertson *et al.*, 1946). All these features occurred in the experimental group on the folate deficient ration during the present study. Individual chicks differ markedly in their tendency to develop clinical manifestations of folate

deficiency (O'Dell and Hogan, 1943) and the body weights and RBC counts of several experimental chicks were still within the control range at 4 weeks of age. Whole blood folate determinations showed, however, that even these chicks had extremely low values as compared with the control group. The whole blood folate values obtained in the present study for the control group are considerably higher than those reported by Cropper and Scott (1966) without conjugase treatment; the reason for this discrepancy appears to be that the level of folic acid in their chick diet (0·3 mg./kg. diet) was, as they state themselves, " barely deficient ".

The microchemical determinations of the total RNA content of individual Purkinje cells gave a mean value of 269 $\pm$ 4 pg. per cell pair, with a range from 251–293 pg., for the control group. Only 1 of the 11 folate deficient chicks had a Purkinje cell RNA content within the control range. The mean value for the experimental group, 217 $\pm$ 8 pg., is about 20 per cent lower than the control value, the difference being statistically significant ($P < 0{\cdot}001$). The variation within the experimental group was comparatively large. The low Purkinje cell RNA values were apparently not only secondary to the presence of anemia as they also occurred in birds with normal erythrocyte and haemoglobin values. It is also difficult to explain the low Purkinje cell RNA values as only due to retardation of body growth, since the mean Purkinje cell RNA value of the folate deficient chicks was significantly ($P < 0{\cdot}01$) lower than that of 3-week-old control animals corresponding to the smallest experimental animal in their body weight. Thus it may be concluded that severe folate deficiency in chicks results in a decreased Purkinje cell RNA content, and that the reduction in neuronal RNA may occur independently of anemia and retardation of body growth. In view of the important role of folates in purine synthesis and the intensity of neuronal RNA metabolism, a likely explanation for the decreased neuronal RNA content would be that folate deficiency interferes with neuronal RNA synthesis.

No clinical signs attributable to cerebellar disturbances or other definite neurological signs could be detected in the folate deficient chicks, except for marked lethargy of early onset. However, it may be of interest in this context to note that a characteristic cervical paralysis has repeatedly been described as a hallmark of the folate deficiency syndrome in turkey poults and goslings, and it occurs occasionally even in chicks (*e.g.* Richardson *et al.*, 1945; Briggs, Hill and Canfield, 1953). Light microscopical studies have not revealed any consistent morphological lesions in the nervous system of folate deficient chicks (Shaw and Phillips, 1945; Haltia, unpublished).

With reference to human pathology it is interesting that a reduced neuronal RNA content of Purkinje cells was found in the folate deficient chicks. Recent observations have necessitated a reappraisal of the significance of folate coenzymes in the nervous system. Various neurological (Hansen, Nordqvist and Sourander, 1964; Long, Childress and Bond, 1963; Anand, 1964; Grant, Hoffbrand and Wells, 1965; Fleming and Dada, 1966; Wells and Casey, 1967; Dérot, Castaigne, Morel-Maroger and Leclercq, 1967) and psychic (*cf.* Strachan and Henderson, 1967) disorders have been reported to occur in human subjects in association with evidence of folate deficiency. Three separate congenital enzyme deficiencies in folate metabolism have been described each of which resulted in infants with mental retardation and dilatation of the cerebral ventricles. Similar findings have been noted in infants reported to have a specific defect in folate absorption (*cf.* Herbert, 1968). Low serum and CSF folate concentrations have been observed

in a large proportion of epileptic patients treated with drugs and it has been suggested that both the therapeutic action and the harmful neurological and psychic side effects of anticonvulsants may depend on some kind of interference with folate metabolism (*cf.* Reynolds, 1968). Furthermore, folates have been observed to concentrate selectively in the cerebrospinal fluid (Herbert and Zalusky 1961), in fact more than most other known substances (Davson, 1967). Finally, the most reasonable explanation available at present for the influence of vitamin B_{12} on nucleic acid synthesis in mammalian cells, including nerve cells (Gomirato, 1954), is that vitamin B_{12} acts indirectly by regulating the level of tetrahydrofolate available to tissues (Buchanan, 1964).

The observations cited above indicate that folates may have a functional role in the human nervous system, and that folate deficiency may have deleterious effects upon it. The present demonstration of an impaired neuronal RNA metabolism in folate deficiency in the chick raises the question whether folate deficiency has a similar effect in mammalian species as well.

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