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Abnormal Folate Metabolism in Feed-Related Anemia of Cultured Channel Catfish (42223)

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Abstract. "No-blood disease" is a severe, feed-related anemia of channel catfish. Pathological features in advanced cases include almost total absence of circulating red blood cells, hepatic fatty change, megaloblastic arrest of hematopoiesis, and intussusceptions of the small intestine. In view of similarities to folate deficiency in man studies were made regarding the intake and metabolism of folic acid. When a bacterial culture medium, containing inorganic salts and folic acid as the sole carbon source, was inoculated with a small sample of anemia-producing feed, over 95% of the folate was destroyed. A yellow precipitate formed and had the characteristic uv spectrum of pteroic acid; it represented 32% of the folate originally present. Differential microbiological assays revealed that the same anemia-producing feed contained 20 times as much folate activity for *Streptococcus fecalis* as for *Lactobacillus casei* (59 μg/g vs 2.6 μg/g). This growth response is compatible with an excess of pteroic acid and/or formyl-pteroic acid which support the former but not the latter organism. Plasma folate activity (mean ± SD, ng/ml) assayed with *L. casei* and *S. fecalis* in 22 normal catfish (hematocrit range 32–43) was 17.2 ± 6.2 and 23.4 ± 13.8, respectively. Comparable values in 15 anemic catfish (hematocrit range 0 to 30) were 35.5 ± 33.7 and 58.7 ± 76.5. The mean plasma pteroate activity, estimated by subtraction, was 6.2 and 23.2 ng/ml, respectively, in normal and anemic fish. Fingerling catfish raised under controlled conditions on feed containing 130 mg/kg of pteroic acid failed to gain weight and developed anemia with the characteristic red cell morphologic features that are seen in the naturally occurring disease. We conclude that severe anemia in channel catfish can be caused by abnormal folate metabolism and may be due to ingestion of folic acid-breakdown products, such as pteroic acid. It is postulated that microorganisms in contaminated feed synthesize folate which, in turn, is converted to pteroate by a pseudomonad or similar organism.

Pond cultivated channel catfish (*Ictalurus punctatus*) in the Southeastern United States are sometimes affected by a severe anemia of unknown cause. "No-blood disease" is the trivial name that has been given to the disorder by commercial catfish farmers. Up to 10% of the stocked population die, but it is believed that growth and development are impaired even in the mildly affected fish that survive. Outbreaks are more common in the spring, and have been described in one pond while an adjacent pond is unaffected. The Alabama Agricultural Experiment Station at Auburn University has records indicating at least 70 occurrences of severe anemia in 1983, and a similar number in 1984 (1) resulting in losses to farmers, processors, and feed manufacturers estimated at several million dollars.

The epidemiology of the disorder has been described (2). No consistent evidence has been found for a viral, bacterial, or parasitic infection, or pesticides, heavy metals, peroxidases, and known mycotoxins as causes of the disorder. However, when farmers empirically changed to a different brand of feed, used a fresh lot of feed, or stopped feeding altogether, the fish stopped dying.

Moribund fish with the typical advanced features of the anemic syndrome have gills which are pale pink, or almost white, rather than the normal deep red color. Blood obtained by venipuncture may have a hematocrit of only 1 to 5% compared to the normal average of about 38% (range 30–50). Apparently healthy fish from an affected pond have been observed with hematocrits of 20–25%. Cases have been observed in which the hematocrit was essentially zero indicating that the anemia is well tolerated. At autopsy the kidneys and spleen are light pink, rather than the normal dark red color; the liver is yellowish gray rather than dark brown and shows fatty change on histologic section. Imprints and smears of the head kidney, which is the major hematopoietic

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organ in catfish, show maturation arrest of red cell formation. Megaloblastic features, strikingly similar to those in bone marrow from human patients with deficiency of folic acid or vitamin B-12, are present in severely affected fish. Another remarkable feature of the disorder is the occurrence of intussusception of the intestine in many of the specimens. In some fish as many as three separate points of intussusception have been identified.

Plumb *et al.* (unpublished) demonstrated that the anemia is feed-related under controlled experimental conditions. Fish fed 1-year old feed that had previously been associated with an outbreak of the problem had an excessive mortality rate and lower hematocrits than controls. Hematocrits as low as 2% were observed in moribund fish from the test cages on the 14th and 21st days, while the minimum hematocrit was 31% in the controls. All surviving fish began to recover when the suspect feed was replaced with normal feed. This experiment demonstrated that the anemia is feed-related, but at the time it was not known if the effect is due to the presence of a toxic factor, or to the absence of an essential nutrient. In considering possible mechanisms, it was recalled that a strain of *Pseudomonas,* described by Levy and Goldman (3), is capable of splitting folic acid into pteroic acid and glutamic acid. This organism, which was first isolated from creek mud, can derive all of its carbon, nitrogen, and energy requirements from the glutamic acid component of folate, while pteroic acid is discarded as an insoluble yellow precipitate. This organism (ATCC No. 25301) has been in use in our laboratory for a number of years providing a convenient source of pteroic acid for use in the chemical synthesis of pteroylpolyglutamates by the solid-phase method (4).

We wish now to describe a series of investigations which suggest that feed contaminated by certain microorganisms under improper conditions of storage contains excessive amounts of pteroic acid, and that this molecule interferes with normal folate metabolism in catfish.

Material and Methods. Specimens of blood and tissue from normal or anemic (hematocrit <30) channel catfish were obtained from the Alabama Agricultural Experiment Station at Auburn University and the Fish Farming Service Center at Greensboro, Alabama. The diagnosis was based on typical epidemiologic and necropsy findings, as well as the absence of other known diseases. Affected fish weighed 200 to 800 g. Normal fish weighing 400 to 1200 g were obtained as controls from the same sources as well as a processing plant. Paraffin sections were made from liver and spleen fixed in 10% buffered Formalin or Bouin's fixative. Peripheral blood specimens were obtained by puncture of the caudal vein using a Vacutainer ® system and evacuated tubes containing EDTA or heparin. Microhematocrits were performed by the method of Larsen and Snieszko (5). Plasma was removed from blood specimens after low speed centrifugation and stored at −4°C. Plasma albumin was measured by the bromcresol green binding reaction of Rodkey (6), (American Monitor Albumin Reagent System, Indianapolis, Ind.). Vitamin B-12 assays were performed on serum or EDTA-plasma by the competitive isotope binding method (Corning Immo Phase TM, Medfield, Mass.).

Microbiological assays. Samples of feed suspected of containing an anemia-producing factor, and samples of normal feed were homogenized in a Polytron homogenizer in either phosphate buffer (pH 7.4), or acetate buffer (pH 4.5) in a ratio of 1:10 (w:v). Samples were assayed before and after incubation with an extract of chick pancreas (Difco Laboratories, Detroit, Mich.). Both buffers contained 1% ascorbic acid to protect labile tetrahydro derivatives. Microbiological assays with *L. casei* (ATCC No. 7469) were performed by the aseptic addition method of Herbert (7), except that feed extracts were boiled 5 min before processing to inactivate possible contaminating microorganisms. Folate activity was determined in reference to a standard curve using pteroylglutamate. Assays with *Streptococcus fecalis* (ATCC No. 8043) were carried out in similar fashion using methods previously described (8, 9). Again folate content was expressed in reference to a standard curve prepared with pteroylglutamate because of the known heterogeneity of folic acid active compounds in the extract. However, standard curves with pteroic acid were regularly prepared to confirm its ability to support growth of the organisms. Uniform lots of dehydrated assay medium (Difco) were employed for both

Lactobacillus casei and *S. fecalis* assays. Plasma samples were assayed in the presence of 1% ascorbate to protect against oxidation of labile forms.

Tests for enzymatic hydrolysis of folate. Twenty-five-milliliter Erlenmeyer flasks containing 50 mg of folic acid and simple inorganic salts (potassium phosphate, calcium chloride, ferrous sulfate, manganese sulfate, and sodium molybdate) were prepared as described by Levy and Goldman (3). After inoculation with approximately 400 mg of dry catfish feed the flasks were gently agitated aerobically in a Dubnoff shaker at 30°C for 24 to 48 hr. The yellow precipitate was collected by centrifugation, washed with water and redissolved in 0.1 *N* NaOH. The ultraviolet spectrum was recorded manually (using a Hitachi uv-visible spectrophotometer, Model 100-40) and compared with an authentic sample of pteroic acid. Quantitation was carried out by calculation of molar extinction coefficients. Test tubes containing 10 ml of the culture medium were inoculated with intestinal contents obtained on a sterile wire loop from four freshly killed fish. Tubes were loosely stoppered with a cotton plug and agitated gently for 48 hr at 30°C.

Aquarium feeding experiment. Catfish fingerlings from a single hatching were acclimated for 1 week to an aquarium environment with an ambient room temperature of 24°C, aerated water temperature averaging 21 to 24°C, and a water flow of 2 liters/min. Prior to starting the experiment the average weight per fish was 23.8 g. The baseline microhematocrit (mean ± SD) determined in eight animals was 38 ± 5.7%. One hundred fingerlings were then randomly allocated to each of two aquaria; one received feedings of standard pelletized Auburn "station" feed,[1] while the other received the same feed that had been ground, mixed with pteroic acid in a ratio of 130 mg/kg and repelletized. Feed was administered daily at a rate of 3% body wt, adjusted periodically according to the population remaining. Five fish were randomly netted from each tank for evaluation after 7 and 14 days. Thereafter, 10 fish were randomly sampled from each tank at 21, 28, 35, and 42 days for hematologic and histologic evaluation. After pithing, the tail was cut off just behind the anal fin; blood was obtained from the transected caudal vein for the microhematocrit, peripheral blood smear, and assays for folic acid. Imprints were made of the head kidney, and histologic sections were prepared of liver and spleen in three to five animals from each group.

The feeding experiment was terminated at the end of 6 weeks because of failure of the water dechlorination system.

Hematology. Smears of peripheral blood stained with either Hemal or Wright's stain were examined for morphologic features. In addition to the 22 normal and 15 anemic specimens (see below), smears were evaluated from 100 fish including those in the earlier cage-feeding experiment of Plumb *et al.* (in review) 20 smears read as unknowns from a separate outbreak and 20 from the pteroic acid feeding experiment. Similarly stained smears and/or imprints from the head kidney were examined in many cases. Standard cytologic features of erythrocytes (such as: size, shape, and staining characteristics of nucleus and cytoplasm; inclusion bodies, cytoplasmic basophilia, hypochromia, vacuolation, hemoglobin content, and maturation) were classified on an arbitrary scale of 1 to 4 according to increasing degrees of abnormality for subsequent correlation with the hematocrit. No attempt was made at this time to evaluate leukocytes or thrombocytes. Reference was made to published results of studies of normal catfish (11) as well as to control specimens collected for the present study.

Results. *Hematology.* Representative features of red cell morphologic abnormalities associated with the anemia are shown in Figs. 1 and 2 along with normal cells for comparison. In general the nuclei of red cells from anemic fish were larger, more rounded, and had a finer chromatin pattern than the compact oval nuclei from normal cells. Cytoplasmic basophilia was common, often accompanied by vacuolation, perinuclear pallor, or clumping of hemoglobin around the periphery of the cell. Both microcytes and mac-

[1] Auburn Station feed is a pelletized ration prepared by a commercial manufacturer to specifications based on published standards. A vitamin mix is added so as to provide known requirements including folic acid at a concentration of 5 mg/kg of dry ration (10).

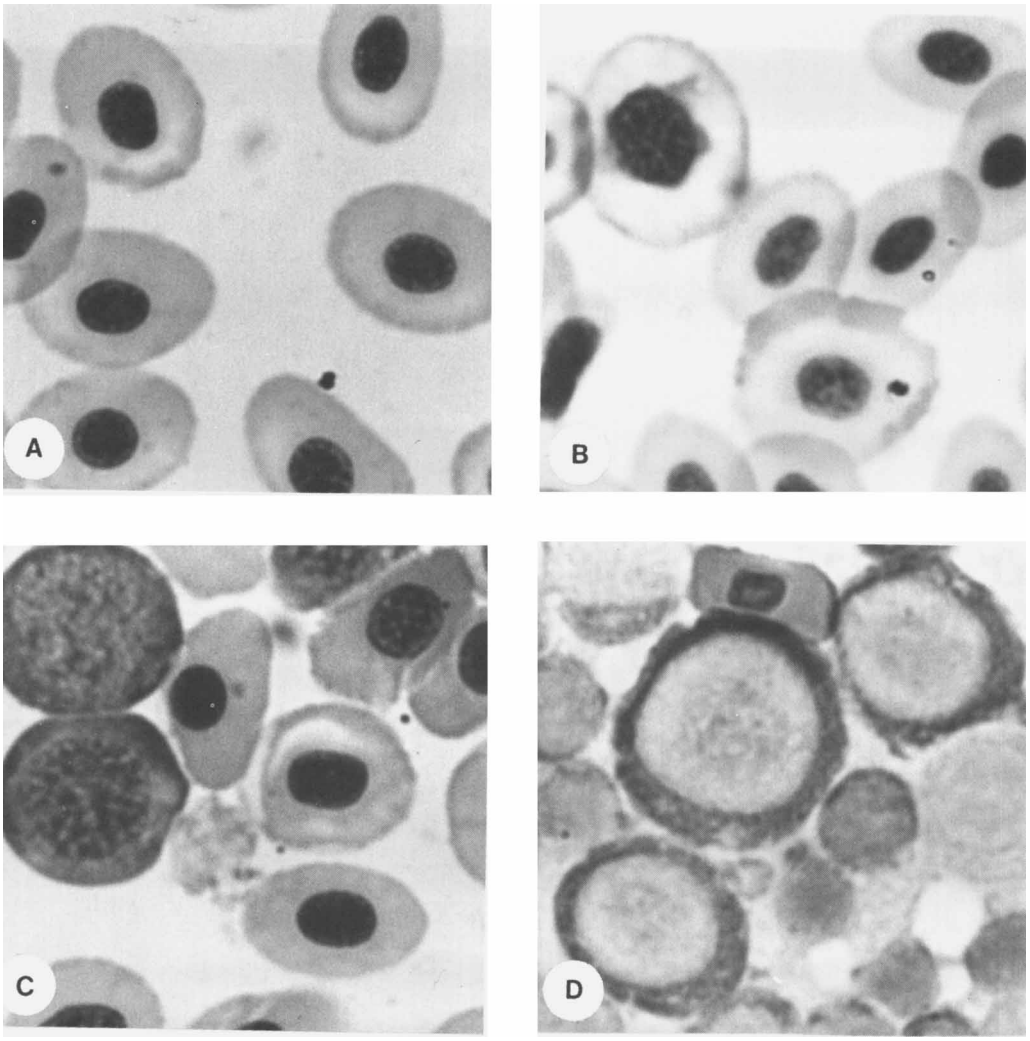

FIG. 1. Blood smears and imprints of head kidney (×1850) (A) Normal peripheral blood. (B) Peripheral blood from an anemic catfish in a natural outbreak of the disease. (C) Normal head kidney showing a young and a slightly more mature hemocytoblast along with several mature, hemoglobinized red cells. (D) Head kidney, naturally occurring disease, showing maturation arrest of hematopoiesis.

rocytes were seen, the larger cells tending to be more immature and basophilic. Occasional primitive cells believed to be hemocytoblasts were found in peripheral blood smears from severely anemic fish. A striking manifestation not seen in normal smears was a partially divided cell connected by a strand of chromatin. Such cells, presumably reflect derangement of the mitotic process; they appear to be identical to those described by Smith (12) in folate deficient salmon.

Imprints of the head kidney from anemic fish revealed increased numbers of hemocytoblasts (Fig. 1D). These were approximately 16–18 μm in diameter and had intensely basophilic cytoplasm with pale nuclei. Although somewhat smaller than megaloblasts of humans, the pattern of distribution of scattered, dark-staining rings in the anemic catfish head-kidney bore a remarkable resemblance to bone marrow smears of humans with pernicious anemia or tropical sprue, especially when

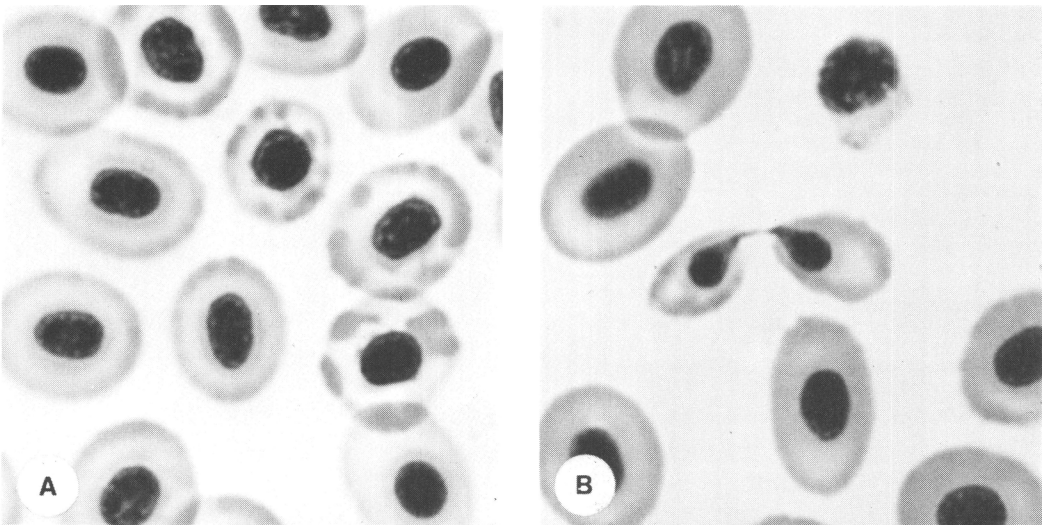

FIG. 2. Peripheral blood from two catfish raised on 1-year-old feed previously associated with a natural outbreak of anemia: (A) Red cells showing cytoplasmic vacuolation and clumping of hemoglobin. (B) Hemoglobinized red cell showing incomplete separation of daughter nuclei ("spectacle cell").

viewed under low magnification. Metaphase plates were observed with evidence of chromosome coalescence and degeneration.

Tests for enzymatic hydrolysis of folic acid. There was an apparent loss, inactivation, or conversion of a portion of the folic acid with each inoculum (Table I). This might be explained in part by adsorption to fiber, or metabolic utilization of folate by unknown organisms surviving with the aid of residual nutrients. However, only the old feed from farm D, which appeared moldy and had previously been associated with a natural outbreak of anemia, yielded a significant amount of pteroic acid as the end product of conversion. When the yellow precipitate which formed was washed with water and redissolved in 0.1 *N* NaOH it gave the identical uv spectrum as authentic pteroic acid. (The observed and expected optical density ratios, respectively, were 2.94 and 2.95 at 256 vs 365 nm; they were 1.04 and 1.12 at 256 vs 277 nm). The 32.3% yield of pteroic acid compares favorably with the yields of 30% by bacterial growth, and 24% by purified enzyme as reported by Levy and Goldman (3). The storage bin used for feed on Farm "D" was encrusted with grayish, fuzzy-appearing particles of feed, a possible source of recontamination of new feed stock.

TABLE I. EXTENT OF FOLIC ACID DEGRADATION AND PTEROIC ACID FORMATION BY MICROORGANISMS IN CATFISH FEED

	Folic acid		Pteroic acid	
Food source	μmol remaining	% degraded	μmol formed	% yield
Farm D, old	<5.00	>95	36.5	32.3
Farm D, fresh	44.5	39	11.8	10.5
Auburn, fresh	43.2	38	7.0	6.2
Farm O, fresh	41.1	36	7.2	6.4

Note. Amount of folic acid at time of inoculation: 113 μmol. Time of cultivation: 48 hr (at 30°C with shaking).

Three of the four test tubes inoculated with intestinal contents from freshly killed fish in a group having anemia developed the typical yellow precipitate that is seen when pteroic acid is formed by a known inoculum of the folate-splitting strain of *Pseudomonas.*

Folate content of feed. Results of assays for folate activity of feeds are presented in Table II. Commercially prepared pelletized catfish feeds are customarily enriched with vitamin additives so as to contain 5 mg of folic acid per kilogram (10). It is believed that adsorption to cellulose, or other insoluble dietary components may have impaired recovery of this

TABLE II. FOLIC ACID ACTIVITY OF CATFISH FEED, μg/g

	L. casei		*S. fecalis*	
	Free[a]	Total	Free	Total
Auburn, fresh stock	0.83	2.39	2.5	14.8
Farm O, fresh stock	1.03	2.25	5.0	14.8
Farm D, fresh stock	1.0	2.40	6.5	17.3
Farm D, old stock	1.7	2.60	47.8	59.0
Auburn stock, treated[b]	—	—	32.5	56.0

[a] "Free" and "total" folate refer to values obtained before and after conjugase treatment at pH 7.4. Similar values, not shown, were obtained at pH 4.5.

[b] Approximately 100 g of fresh Auburn stock were mixed with 10 g of Farm D old stock, moistened and allowed to stand in a glass dish at room temperature for 48 hr redryed and assayed as before.

added folate, which under ordinary circumstances would be expected to support both *S. fecalis* and *L. casei* equally. In all cases the growth-supporting activity is somewhat greater for *S. fecalis* than for *L. casei*. This is best explained by the presence of pteroic acid and/or formyl-pteroic acid (rhizopterin) which have been known for many years to support *S. fecalis* but not *L. casei* (13). Both organisms respond almost equally well to unsubstituted folic acid and to folinic acid (*N*-5-formyltetrahydropteroylglutamate), but only *L. casei* reflects the presence of *N*-5-methyltetrahydropteroylglutamate.

In other experiments (unpublished) even a 10-fold excess of pteroic acid does not inhibit the ability of *L. casei* to respond to pteroylglutamate under standard assay conditions. In terms of growth supporting ability for *S. fecalis* approximately 1.5 moles of pteroate are equivalent to 1.0 mole of folate. One sample of feed that had been associated with a severe outbreak of disease contained 20 times as much folate activity for *S. fecalis* as for *L. casei*. When this contaminated feed was moistened and added to normal Auburn feed and allowed to stand 48 hr at room temperature, a marked increase in *S. fecalis* activity occurred (Table II).

Laboratory indices of nutritional status of pond-raised catfish. The results of folate assays with two organisms, and the determination of hematocrit, plasma B-12, and serum albumin are presented in Table III.

The low hematocrit values in anemic fish are attributable in part to use of the hematocrit in making the diagnosis. However, the low mean value reflects the range that is seen in fish with other characteristic features of the disease such as hepatic fatty change, pallor of internal organs, and intestinal intussusception.

Significant lowering of plasma albumin ($P < 0.01$) occurred in anemic fish. While this could be due in part to hemodilution it is perhaps more likely the result of hepatic dysfunction and/or inadequate protein intake. Similar values for circulating vitamin B-12 in both groups suggest that vitamin B-12 deficiency is not a factor of primary etiological significance.

The wide range of variability in plasma folate content may be attributed to irregular patterns of feeding in the natural environment. As noted in the introduction, fish apparently recover if feed is withheld. Thus, toxicity appears to be cumulative but partially reversible, so that irregular feeding patterns may account for wide variations in folate activity of plasma from affected fish. In any event the total plasma folate activity tends to be considerably higher in anemic than in normal fish. This is statistically significant ($P < 0.01$) for the *L. casei* assay, but somewhat less so ($P > 0.05$, <0.10) for *S. fecalis* owing to wide variability in the latter. Even in normal fish there is an

TABLE III. LABORATORY INDICES OF NUTRITIONAL STATUS OF POND-RAISED CATFISH

	Normal mean ± SD (n)	Anemic mean ± SD (n)
Hematocrit, %	37.8 ± 3.2 (22)	11.2 ± 12.7[a] (15)
Plasma albumin, g/dl	1.37 ± 0.26 (22)	0.79 ± 0.59[a] (15)
Plasma *L. casei* activity, ng/ml	17.2 ± 6.2 (22)	35.5 ± 33.7[b] (15)
Plasma *S. fecalis* activity, ng/ml	23.4 ± 13.8 (22)	58.7 ± 76.5[c] (15)
Plasma vitamin B-12, ng/ml	18.38 ± 10.18 (14)	17.72 ± 8.66 (14)

Note. Superscripts indicate probability of significant difference from normal by the unpaired *t* test: [a]<0.001, [b]<0.01; [c]>0.05 <0.10.

TABLE IV. CATFISH, AQUARIUM FEEDING EXPERIMENT

	Hematocrit, %, mean ± SD		Weight, g, mean	
	Controls	Pteroic fed	Controls	Pteroic fed
Initial (n = 8)	38.0 ± 5.7	—	23.8	
7 days (n = 5)	35.0 ± 2.0	32.8 ± 5.0	ND	ND
14 days (n = 5)	40.2 ± 2.0	38.4 ± 5.8	27.0	19.5
21 days (n = 10)	39.4 ± 3.9	32.6 ± 3.5*	28.5	20.7
28 days (n = 10)	35.2 ± 5.8	31.9 ± 4.8	27.7	22.8
35 days (n = 10)	34.0 ± 4.2	31.8 ± 6.9	26.0	22.8
42 days (n = 10)	36.7 ± 4.3	29.2 ± 4.5*	29.5	23.7

* Hematocrit values are significantly lower in pteroic-fed animals on Day 21 and Day 42 ($P < 0.01$, unpaired t test), and on the aggregate sample Day 21 through 42, ($P < 0.001$, unpaired t test), than in controls. Initial weight of control fish based on a batch of 20. ND, not done. Identical values for average weight of pteroic-fed fish on Days 28 and 35 confirmed.

average excess of 6 ng/ml of folate activity for *S. fecalis* as opposed to *L. casei,* presumably due to the presence of pteroic acid and/or formyl-pteroic acid. In anemic fish this discrepancy rises to approximately 23 ng/ml.

Feeding experiment. Results of the pteroic acid feeding experiment are shown in Table IV. At each sampling time the weight and hematocrit were lower in the fingerlings on the pteroate-containing feed than in their counterparts who received normal feed. Statistical comparisons were not feasible on the weights which were determined in batches, although it is apparent the pteroic-fed fish failed to grow. At the end of the experiment hematocrit values were significantly lower ($P < 0.01$) in treated animals than in controls, by the unpaired t test. The difference was even more pronounced ($P < 0.001$) on the aggregate sample comparing all hematocrits of treated animals with controls from Day 21 through Day 42.

Blood smears from the 10 pteroic-fed and the 10 control-fed fish sacrificed at the sixth week were read as unknowns by a single trained observer. Five of the pteroic-fed fish had hematocrits of 30 or less; three of these five were identified as having the characteristic morphologic features found in the naturally occurring disease, including partially divided ("spectacle") cells in the peripheral blood and head kidney (Fig. 3). None of the control fish displayed these features. Histologic sections of liver from pteroic-fed fingerlings did not show fatty change in the limited number of specimens available. It remains to be seen if this lesion would occur in a larger sample, or with more prolonged exposure to pteroic acid.

Discussion. In 1963 Johns and Plenderleith reported the ability of pteroic acid to displace folic acid from tissue storage or binding sites in man (14). Injections of unlabeled pteroylglutamate, pteroyltriglutamate, or pteroic acid each caused urinary excretion of 68 to 77% of a previously injected dose of tritium-labeled folic acid. Pteroic acid was even more effective in displacing folate than methotrexate and folinic acid, which displaced an average of 56 and 42.5%, respectively. The authors concluded that the flushing effect was dependent upon structural integrity of the pteridine moiety, since *p*-aminobenzoylglutamate and glutamate were ineffective displacing agents. Earlier work by Franklin, Stokstad, and Jukes (15) had suggested that intravenous administration of pteroic acid to human subjects led to its conversion to pteroylglutamate. However, since pteroic acid cannot correct folic acid deficiency in man (16) it is more likely that the folic acid excretion observed by Franklin and co-workers was also due to displacement of endogenous folate. In view of the fact that pteroic acid is not an effective coenzyme precursor and yet is capable of competing with folate for carrier or binding sites it should be regarded as a folic acid antagonist. It seems likely that the ability of pteroic acid to serve as a growth factor in a unique vitamin assay system, the *S. fecalis* assay, has over the years obscured its capacity to function as a folate antagonist in other biological sys-

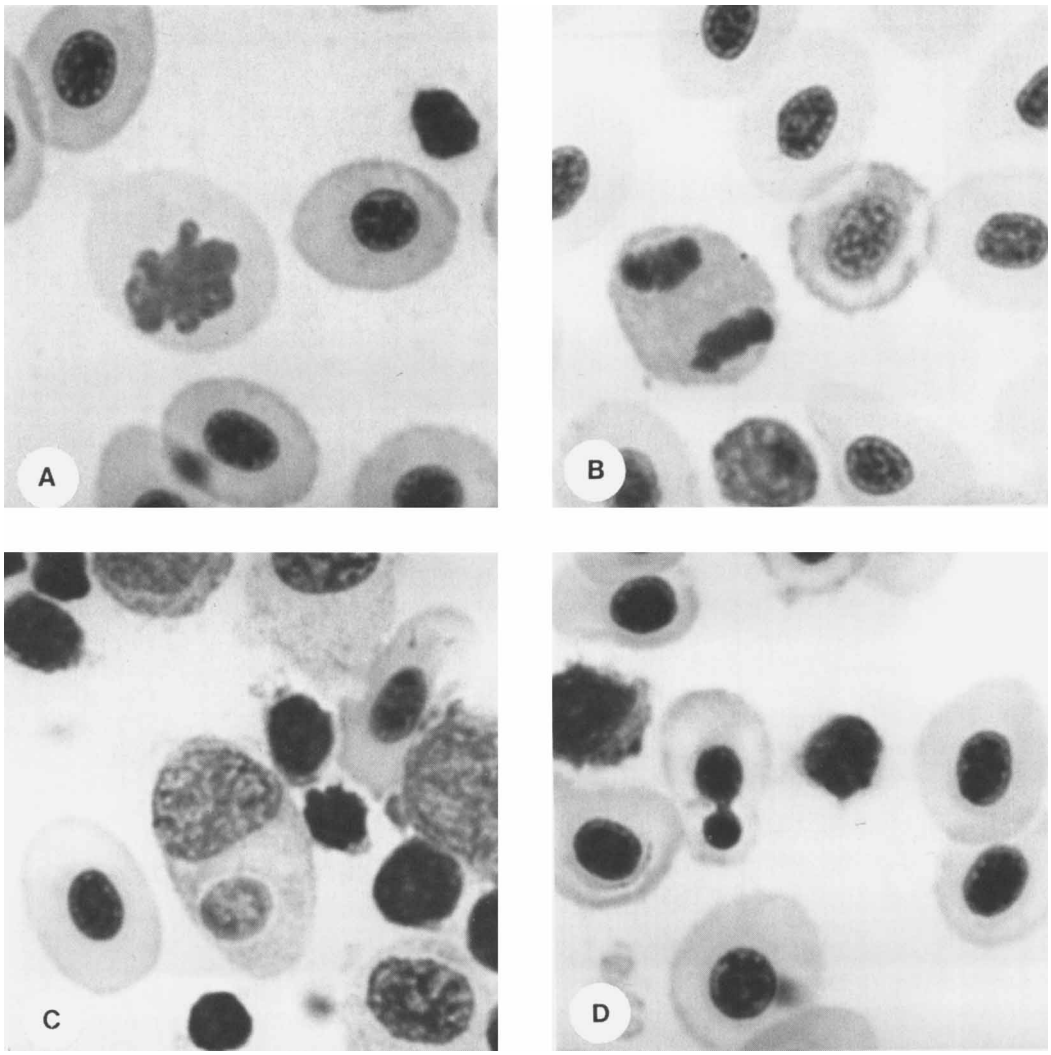

FIG. 3. Peripheral blood and head kidney smears of fingerling catfish after exposure to pteroic-acid-containing feed (×1850). (A) Large red cell with irregular nuclear border, probably a degenerating mitotic figure. (B) Late anaphase figure in peripheral blood; note also cytoplasmic vacuolation and clumping of hemoglobin. (C) Head kidney showing neutrophil with micro- and macronuclei. (D) Peripheral blood showing incompletely divided nuclei ("spectacle cell").

tems. It has been shown that pteroic acid is a competitive inhibitor of dihydrofolic (DHF) reductase (17). It has also been shown that other well-known inhibitors of dihydrofolic reductase, methotrexate and aminopterin, decrease the rate of entry of folic acid into cells (14, 18, 19). The suggestion has been made that this is due to competition for a common transport or carrier mechanism (14). The high circulating folate value observed in anemic fish in the present study is unexplained. While further investigation is needed to clarify this finding, it could be due, in part, to blocking of folate receptors by structurally similar molecules of pteroic acid.

The present study began with the hypothesis that a microorganism was destroying, or inactivating, folate. Therefore, the finding of

high folate values by *S. fecalis* assay of contaminated feed samples suggested more than simple inactivation of folate in the manufactured formula. Our results suggest that additional folate is produced in the feed, perhaps by fungi, and subsequently degraded to a compound that functions as a folic acid antagonist. Certain bacteria are known to be capable of converting folic acid to pteroic acid. Although the feeds associated with outbreaks of anemia in catfish have high numbers of bacteria and fungi, these organisms have not yet been identified. To the best of our knowledge the present study is the first to describe a link between a naturally occurring folate antagonist and an animal disease. These observations raise the possibility of an association between pteroic acid, or other naturally occurring folate antagonists, and other diseases of animals and humans.

The evidence of nuclear injury (Fig. 3) in pteroic acid-treated fish raises questions regarding the potential for mutagenicity and carcinogenicity of pteroate. The role of folate deficiency in carcinogenesis, and the induction of secondary cancers following therapy with folate antagonists have been reviewed by Krumdieck (20).

Pteroic acid has been identified chromatographically in extracts of dark-roast coffee (21) and in cultures of a common soil organism, *Bacillus subtilis* (22), although possibly as a decomposition product. Thus the possibility exists for human exposure to pteroic acid from a variety of sources. The degree of exposure could have a bearing on the nutritional requirement for folic acid.

Fatty degeneration of the liver may have multiple etiologic mechanisms including protein deficiency, antibiotics, or other toxic factors which inhibit lipoprotein synthesis, and deficiency of the lipotropic factors methionine and choline. Fatty liver in humans is often an accompaniment to alcoholism which has been shown to increase nutritional requirements for folate (23). In view of the well-known role of *N*-5-methyltetrahydrofolate as a methyl donor in the biosynthesis of methionine, this pathway may be impaired in anemic catfish as a result of either DHF reductase inhibition or impaired folate transport in the presence of pteroic acid. Other folate-dependent pathways of amino acid metabolism might be hindered by similar mechanisms. Even enzymes requiring biopterin as a cofactor, such as phenylalanine hydroxylase might be susceptible to injury since DHF reductase is required for the formation of tetrahydrobiopterin the active coenzyme.

In summary, evidence has been presented that the feed-related anemia of channel catfish is accompanied by maturation arrest of hematopoiesis similar to that seen with folate deficiency in man. It has been shown that feed associated with outbreaks of the disorder is contaminated with microorganisms capable of converting folic acid to pteroic acid. Differential microbiological assay of contaminated feeds has shown significantly greater folic acid activity for *S. fecalis* than for *L. casei* in comparison with normal feed. This is indicative of the presence of pteroic acid and/or formylpteroic acid (rhizopterin) which support the former but not the latter organism. A similar pattern was observed in the plasma of affected fish. When pteroic acid was incorporated into the feed of fingerling channel catfish under controlled conditions, several major manifestations of the naturally occurring disease were reproduced. These included failure to grow, anemia, maturation arrest in hematopoietic tissue, and morphologic abnormalities in circulating red blood cells. It is concluded that bacteria and/or fungi in contaminated feed synthesize excessive amounts of folic acid compounds (pteroyl-monoglutamates and -polyglutamates) which are degraded to pteroic acid, a naturally occurring folic acid antagonist.

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