AN ABSTRACT OF THE THESIS OF

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Title: SOME ENZYME CHANGES IN LIVERS OF RAINBOW TROUT (SALMO GAIRDNERII) FED AFLATOXIN B_1 AND STERCULIC ACID

Abstract approved: M. W. Montgomery

Initial effects of feeding rainbow trout semi-purified diets containing the cocarcinogen, methyl sterculate, or the carcinogen, aflatoxin B_1 on certain liver dehydrogenase enzymes of glucose metabolism were studied. Activities of lactate dehydrogenase (LDH) of the glycolytic pathway, glucose-6-phosphate dehydrogenase (G6PDH) of the pentose phosphate shunt, and isocitrate dehydrogenase (ICDH) and malate dehydrogenase (MDH) of the citric acid cycle were investigated by spectrophotometric assays on extracts of fish liver homogenates. Vertical polyacrylamide gel electrophoresis was used to study the isozymes of LDH, MDH, and G6PDH of the extracts. Periodic histological observations of the liver tissue were also made.

A diet of aflatoxin B_1 at 20 ppb caused an increase in the specific activity of G6PDH and a decrease in the specific activity of NAD-linked ICDH within 28 days. The specific activity of the other enzymes

remained within the range of values determined for the controls. No morphological changes or variations in isozyme patterns were noted during a 35-day feeding period.

Methyl sterculate, the methyl ester of 2-octyl-1-cyclopropene-1-octanoic acid, was fed at two levels: 100 ppm to 18 week old and 21 week old trout and 200 ppm to 23 week old fish. In the three feeding trials, all five dehydrogenases showed initial decreases in specific activities and in certain cases recovered to normal activity levels after several days. Length of feeding time necessary to cause the decrease in specific activity appeared to depend upon the particular enzyme, the age of the fish, the weight of the liver, and the level fed. G6PDH was the most sensitive enzyme while ICDH activity was affected to a much lesser degree. The younger group of fish showed the effects more quickly but also appeared to recover to normal activity at an earlier stage of feeding than the older fish. The critical factor for the decrease in enzyme activity was probably the level of sterculate actually deposited in the liver. This critical level would vary both with liver weight and the level in the diet. Once the critical level was reached the enzymes would be inhibited. The critical level of sterculate appeared to be different for each enzyme and probably depended on the relative sensitivity of the enzyme to sterculate.

Dietary sterculate also induced certain morphological changes in rainbow trout livers. The livers characteristically became enlarged,

pale, and firm as compared to normal livers. Microscopic examination showed that glycogen deposits began building up within one week of feeding, and later fibrous strands of unknown origin appeared within the cell.

A change in isozyme pattern was noted with G6PDH after feeding sterculate. While one isozyme was present in normal trout liver, two bands of G6PDH activity were found after feeding sterculate. It was suggested that the additional band of G6PDH might be due to an irreversible binding of sterculate to a sulfhydryl group not in the active site which allowed the enzyme to remain active but altered its electrophoretic mobility. No alteration was found in the isozyme patterns of LDH and MDH.

The cocarcinogenicity of sterculate is likely due to an inhibitory action within the cell. This inhibitory action somehow enhances the effect of aflatoxin B_1 in the cell possibly by inhibiting a detoxification mechanism for the aflatoxin.

Some Enzyme Changes in Livers of Rainbow Trout (Salmo Gairdnerii) Fed Aflatoxin B_1 and Sterculic Acid

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INTRODUCTION

In 1960, an epizootic of liver cancer occurred in hatchery-reared rainbow trout. Wolf and Jackson (133) and Sinnhuber et al. (114) traced this outbreak to the contamination of cottonseed meal in the diet by a mold toxin known as aflatoxin. In their search for other carcinogens in the cottonseed meal, Sinnhuber et al. (112, 115) found that cyclopropenoid fatty acids (CPFA) were powerful cocarcinogens with aflatoxin B_1 for the induction of primary hepatomas in rainbow trout. While not carcinogenic themselves, the CPFA did produce certain transitory morphological changes in the liver including the accumulation of glycogen deposits and the development of fibers of unknown origin within the cell (65, 98).

The carcinogenic aflatoxins were first discovered in England in 1961 as contaminants in peanut meal (62) and have since been found as natural contaminants in a number of foods and feeds. These mycotoxins produced by the mold Aspergillus flavus have now been recognized as crop contaminants of considerable economic and nutritional importance. The aflatoxins have been shown to induce hepatomas in rats (11, 62), ducks (128), and guinea pigs (105) as well as trout. In addition, these compounds have been implicated as

causative agents in many epizootics among farm animals (128) and may also be responsible for a liver disease in dogs known as hepatitis-x (82). Coady (28) reported a high incidence of liver cancer among African people known to consume aflatoxin-contaminated food. However, an etiological relationship between aflatoxins and human liver cancer has not been conclusively proven.

The cocarcinogenic CPFA are also quite common constituents in the diets of animals and man. CPFA have been found to occur naturally in the oils of many plants in the order Malvales including the economically important cotton plant. Cottonseed meal containing CPFA have been shown to cause profound effects when fed to poultry including pink discoloration of the egg, decreased growth, depressed egg production, impaired fertility, and an alteration in fat composition (85, 109, 111). Some of these effects have also been found in other species including the trout. As a possible biochemical basis for its activity, CPFA have recently been shown to inhibit certain enzymes including fatty acyl desaturase (91), yeast alcohol dehydrogenase (91), and castor bean lipase (83).

The present study was undertaken to further elucidate some of the in vivo effects of aflatoxin B_1 and sterculic acid (CPFA) on certain enzymes involved in glucose metabolism in rainbow trout livers.

REVIEW OF LITERATURE

Tumor Enzymology

Neoplasia is characterized by a derangement of cellular control mechanisms. This does not imply that those control mechanisms involved in the synchronization of biosynthetic processes and energy production are not retained. However, as Wenner (123) points out, the response to controlling factors, which limit the capacity of the normal cell for unrestricted growth, is altered in malignancy.

On the intracellular level, control mechanisms operate through the regulation of multienzyme pathways. Cellular regulation of the rates of these multienzyme pathways is exerted by controlling the reaction velocities of certain rate-limiting steps. The derangement of control mechanisms associated with neoplasia could be connected with the rates of critical reactions and the enzymes associated with them.

Effect of Cancer on Carbohydrate Metabolism

One of the multienzyme pathways, glycolysis, is strikingly affected by the onset of malignancy. In 1931, Warburg (120) showed that cancerous tissues were characterized by a high rate of lactate formation under aerobic as well as anaerobic conditions. This announcement has resulted in heavy emphasis being placed on comparisons of carbohydrate metabolism and associated enzymes of normal

and neoplastic tissues. Much of the work has been with transplanted tumors, which may vary considerably from the primary hepatomas of rainbow trout used in this study. The literature on transplanted tumors will not be discussed in detail here.

Warburg postulated that respiration, i. e., the citric acid cycle, was impaired in neoplastic cells leading to an increased dependence on glycolysis for energy (123). It has since been shown that the citric acid cycle is fully operative in neoplastic tissues (10, 125) and that the electron transfer chain (20, 21) and oxidative phosphosphorylation (1, 124) were not affected. Emmelot(38) confirmed this observation for ethionine and dimethylnitrosamine-induced primary hepatomas.

Hence, there is a breakdown in the mechanism which regulates the rate of glycolysis in the cell under aerobic conditions (123). Many studies on the enzymes of carbohydrate metabolism have been made in an attempt to determine where the control mechanism breaks down. The question remains unanswered. Four excellent reviews have been written recently concerning this area of research (16, 61, 95, 123).

Some of the proposed control mechanisms concerned with energy metabolism are based on activities of the dehydrogenases. The most extensively studied enzymes in neoplastic tissues are malate, lactate, and isocitrate dehydrogenases. Early studies showed the activity of malate dehydrogenase (L-malate: NAD oxidoreductase, EC 1.1.1.37), designated MDH, of neoplasms to be comparable to that of normal

tissue (125). Several workers have shown that MDH activity decreases by about 50% when rat breast becomes cancerous (52, 93). With transplanted tumors, MDH progressively decreases in hepatomas with increasing growth rate (121). The amount of mitochondria in neoplasms is also decreased (1) and it appears that the lowered MDH activity represents a lowering of the mitochondrial enzyme.

Lactate dehydrogenase (L-lactate: NAD oxidoreductase, EC 1.1.1.27), designated LDH, activities have been shown to be comparable to those of normal cells (93). Other workers have supported this as summarized by Knox (61). Hershey et al. (52) showed that the activity of isocitrate dehydrogenase (L_s-isocitrate: NADP oxidoreductase, EC 1.1.1.42), designated NADP (nicotine adenine dinucleotide phosphate)-ICDH, was increased in rat breast tumors when compared on a wet weight basis, but not significantly changed if compared per weight of DNA-P (deoxyribonucleic acid-phosphorous). McLean and Brown (71) found a decrease in activity in primary hepatomas for NADP-ICDH. Hawtrey (51) reported the presence of NAD (nicotine adenine dinucleotide)-ICDH (L_s-isocitrate: NAD oxidoreductase, EC 1.1.1.41) and NADP-ICDH in primary hepatomas. Although these enzymes were reduced in activity, they still occurred in the same proportion.

Glucose-6-phosphate dehydrogenase (D-glucose-6-phosphotransferase, EC 1.1.1.49), designated G6PDH, is an

important rate-limiting enzyme in the pentose phosphate pathway (95). Higher activity of this enzyme has been found in primary hepatomas induced by feeding azo-dyes (72). In transplanted rat hepatomas, a higher level of G6PDH was found in both the fast-growing Novikoff hepatomas (122) and the slow-growing Morris 5123 hepatomas (89), in comparison to the relatively low normal levels. Significantly, perhaps, higher activity was also found in hyperplastic nodules which may be benign forerunners of hepatocarcinomas (94).

Several other enzymes have received a good deal of emphasis recently. As Knox (61) points out, both hexokinase (ATP: D-hexose-6-phosphotransferase, EC 2.7.1.1) and phosphofructokinase (ATP: D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11) are key enzymes since they catalyze the rate-limiting steps of glycogen breakdown and glycolysis, respectively. Significant increases in activities of both these enzymes were seen in neoplasms (61).

Isozymes and Cancer

Isozymes were defined by Markert and Moller (76) as multiple molecular forms of proteins which can catalyze the same reaction. Multiple molecular forms of enzymes were first found for lysozyme (119) and lactate dehydrogenase (80) with the development of zone electrophoresis techniques, utilizing charge and size differences to separate proteins in a porous matrix. Many more enzymes have been

shown to exist in multiple forms. The most common supporting mediums for electrophoresis have been starch gel (116) and polyacrylamide gel (92).

The genetic and functional significance of the multiple forms has not been clarified for the majority of isozyme systems. For LDH and MDH the structure, genetic origin, and the significance of the tissue distribution of the isozymes has been partially determined (58). Markert (74) has proposed five mechanisms to account for the presence of multiple forms of enzymes. The simplest mechanism would be a single gene responsible for encoding the enzyme, followed by partial degradation of the polypeptide chain to yield multiple forms. Secondly, a single polypeptide chain could polymerize to yield a series of isozymes of different polymer size. Another possible mechanism would be for the enzyme to combine with various small molecules to yield a variety of conjugated proteins. A fourth possibility would be the presence of conformational isozymes. In addition, two or more genes could act to encode different polypeptide chains which would combine to give several isozymes. All of these mechanisms have been postulated to exist in natural systems. For the most part, the physiological significance of the isozymes is unknown, although Stadtman (118) has reviewed some well known examples in microbial systems and Kaplan (58) has reviewed the findings for LDH and MDH.

In mammals, five isozymes of LDH have been found and are

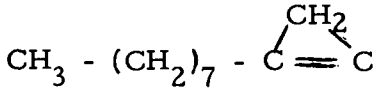
thought to be the five possible tetramers formed by combining two genetically distinct subunits (74). The two subunits were designated H (heart) and M (muscle) after the tissues in which they predominantly occur. In fish, one to eighteen isozymes have been reported (75). Rainbow trout have been shown to contain nine isozymes of LDH (9, 13, 14), although 15 (79), 6 (43), and 4 (44) have also been reported. Goldberg (44) found nine isozymes of LDH for brook trout (Salvelinus fontinalis) and postulated that 15 were actually present on the theoretical combination of three subunits. Bailey and Wilson (9) proposed on the basis of various biochemical and immunochemical results that trout had two separate gene loci for the H subunit. They postulated the theoretical existence of 15 isozymes and, while finding only nine, suggested that some might have identical electrophoretic mobilities. This work was partially confirmed by Massaro and Markert (77), who suggested that salmonids were tetraploids containing twice as much DNA and twice as many chromosomes as higher vertebrates. Trout liver has been reported by Massaro and Markert (77) to have three isozymes of LDH. Bouck and Ball (14), using a much longer incubation period, found six isozymes of LDH to be present in trout liver. However, due to the long incubation time some of these bands could have been artifacts.

LDH isozymes have been used to identify discrete damage in the tissues of higher vertebrates (64). Various pathological conditions result in a release of LDH into the blood stream which alters the

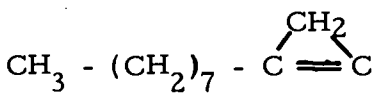
isozyme distribution in the plasma. In early work on hepatomas in rainbow trout, Scarpelli et al. (100) observed a deviation from normal in the plasma LDH pattern using paper electrophoresis. In later work with rat hepatomas, Johnson and Kampschmidt (57) showed that the plasma LDH zymogram of the host was altered as the tumor became larger until it closely resembled that of the tumor itself. These workers found that different tumors had different isozymic patterns and the tumor patterns did not resemble those of the tissue of origin. This was found for both the Novikoff transplanted tumor and 4'-methyl-4-dimethylaminobenzene-induced primary hepatoma. From this work, speculation has been raised that the change in LDH isozymes may be one of the earliest alterations when a tissue becomes malignant and this change in LDH may influence the rate of glycolysis. Langvad (63) has supported this by finding the LDH pattern of tumor-bearing colon in humans varied from normal tissue as a function of the distance from the tumor. In a study of rat breast cancer, Hershey et al. (52) found that the isozymic patterns of both LDH and MDH were altered in malignancy. They found the patterns of G6PDH were unchanged.

Cyclopropenoid Fatty Acids

The two most common cyclopropenoid fatty acids (CPFA) that exist in nature are sterculic and malvalic acids. Sterculic acid is a C-19 fatty acid with the following structure:



Malvalic acid is a C-18 fatty acid having the structure shown below:



Oils from the seeds of 45 plants in the order Malvales and one in the order Ebernales have been reported to contain these compounds in their triglycerides (109). Cottonseed oil is the most economically important oil containing these acids and contains 0.7 - 1.5% malvalic acid and 0.3 - 0.5% sterculic acid (108). Experimental sources of these acids include Sterculia foetida oil (49% sterculic acid, 7% malvalic acid) and Hibiscus syriacus oil (2% sterculic acid, 19% malvalic acid) (65).

A common detection method for cyclopropenoid compounds is the Halphen test developed in 1897 (45) and quantitatively modified by Deutchman and Klaus (32) and Bailey et al. (8). The latter method has been applied to trout lipids (29, 98). Another possible detection method is the modified hydrogen bromide titration method (49, 50, 73). Quantitative analysis of the individual CPFA is rather complicated and no one method is in general use. Two good reviews on the isolation and estimation of these compounds exist (85, 109).

The chemistry of the CPFA is beyond the scope of this study, and therefore will not be reviewed in detail here. An excellent review on the chemistry of these two acids has been published by Carter and

Frampton (19).

The feeding of cottonseed has been implicated in a wide variety of physiological disorders, which have now been attributed to the presence of CPFA. These effects are well documented in three recent reviews (85, 109, 111). A pink discoloration of egg whites was attributed to the feeding of cottonseed meal as early as 1928 (110) and later to CPFA (78, 108). A cessation of egg laying (108), a severe decrease in hatchability (104), and a delay in the sexual development of chickens (103) and rats (107) have been attributed to CPFA in the diets. It is interesting to note that mice do not show the delay in sexual development (109). Ingestion of these acids slows the growth of chickens (101), rats (102), and rainbow trout (98).

Another biological effect of feeding CPFA is an alteration of lipid metabolism which leads to a higher stearate:oleate ratio. This effect has been found in hens (5, 40), pigs (37), rats (96), and rainbow trout (29, 98). Recently, Raju and Reiser (91, 96), Johnson et al. (56), and Allen et al. (40) have attributed the above effect to an inhibition of stearic fatty-acyl desaturase by CPFA. On the basis of kinetic data, Johnson et al. (56) concluded that the inhibition was competitive and due to structural similarities between substrate and inhibitor. Raju and Reiser (91) contended, however, that the inhibition is non-competitive, and is due to the reaction of the cyclopropene ring with sulfhydryls. This contention was supported by Kircher (60) who

demonstrated reactions between methyl sterculate and various sulfhydryls *in vitro*. Also, Ory and Altschul (83) have shown that sterculic acid could inhibit the action of lipase (glycerol ester hydrolase, EC 3.1.1.3) which has sulfhydryls at the active site. Some reversal of this inhibition was demonstrated by addition of cysteine to the system. In further work, Raju and Reiser (91) demonstrated that yeast alcohol dehydrogenase (alcohol: NAD oxidoreductase, EC 1.1.1.1), which has a number of reactive sulfhydryl groups, was also inhibited by CPFA. Holloway *et al.* (53) further supported the latter contention by proposing that fatty-acyl desaturase was a thiol enzyme since low concentrations of a common sulfhydryl reagent, *p*-hydroxymercuribenzoate, inhibited the activity.

The only evidence of any effect of CPFA on carbohydrate metabolism is the inhibition of yeast alcohol dehydrogenase mentioned previously.

In 1966, Sinnhuber *et al.* (115) reported that CPFA enhanced the incidence and growth of aflatoxin-induced hepatomas in rainbow trout A diet containing 200 ppm cyclopropene acids and 4 ppb aflatoxin B_1 produced an 83% incidence of tumors in six months, compared to a complete lack of tumors with aflatoxin alone. Lee *et al.* (65) found similar effects for both sterculic and malvalic acid. CPFA was also shown to be co-carcinogenic with 2-acetylaminofluorene (65).

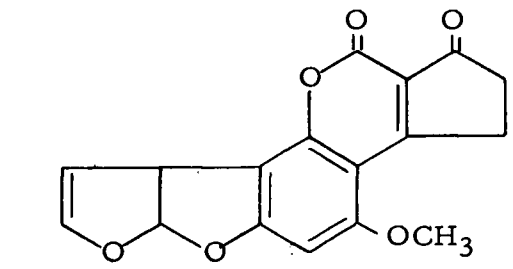
CPFA by themselves were not found to be carcinogenic to trout,

but histological examination revealed extreme liver damage including deposits of glycogen and formation of fibers in the cells (65, 98). The livers were enlarged, firm, and displayed a marked lack of pigmentation (98). Evidence has been gathered by Roehm (98) to show that CPFA were deposited intact in all body tissues and that they accumulated in all tissues studied.

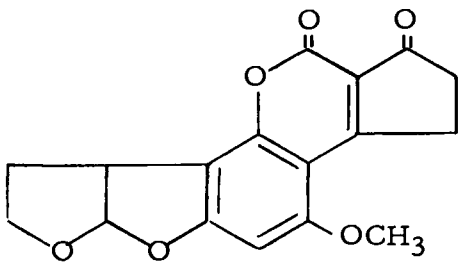
Aflatoxins

Aflatoxins are mycotoxins produced by certain strains of Aspergillus flavus and Aspergillus parasiticus. Eight naturally occurring aflatoxin compounds have been identified as derivatives of the same difuranocoumarin structure as shown in Figure 1 (23). The structures of these compounds were determined by several workers (6, 7, 34, 55). Excellent reviews on mycotoxins have been published (23, 127, 129, 131).

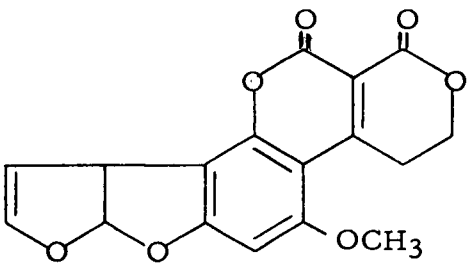
Aflatoxins commonly occur in mold-infested peanuts and cottonseed, but have also been found in other foods and feeds including low grade corn and coconut (23). Aflatoxin M has been reported in the milk of cows (3, 30) and rats (30) and sheep urine (3) when these animals were fed an aflatoxin B_1-containing diet. Occurrence of aflatoxins in other biological systems has been rare although A. flavus has been grown on a variety of agricultural products including cassava, rice, wheat, and oats with good toxin production (23). Lie and Marth (69) found that 3-month-old cheddar cheese would support growth and



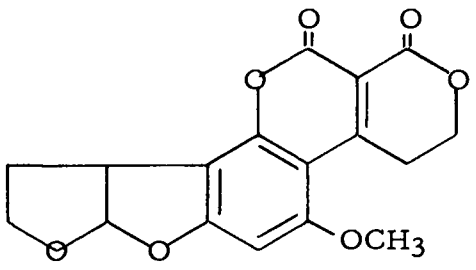
B_1



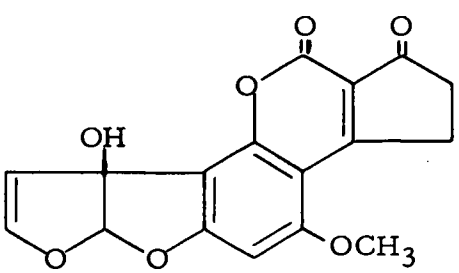
B_2



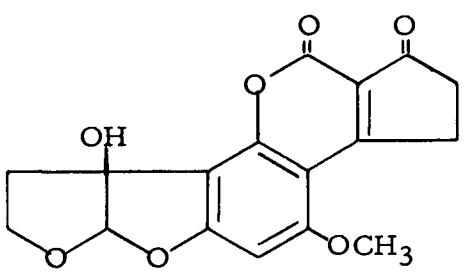
G_1



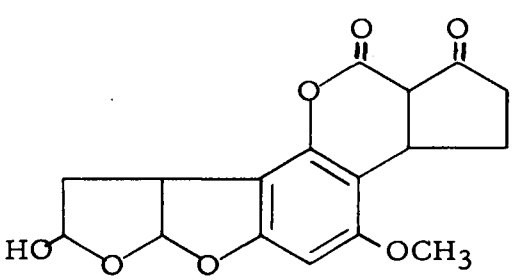
G_2



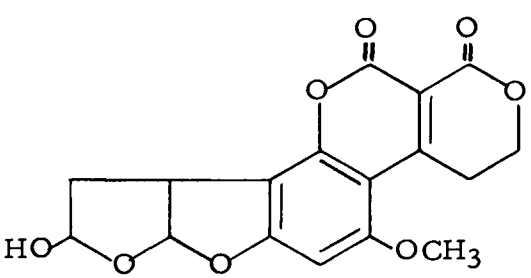
M_1



M_2



B_{2a}



G_{2a}

Figure 1. Structural formulas of the various aflatoxins.

aflatoxin production by A. flavus.

Biological toxicity of the aflatoxins was first demonstrated by Lancaster et al. (62), who reported a high incidence of liver tumors in rats fed for 30 weeks on diets containing aflatoxin-contaminated peanut meal. Subsequently, several epizootics among farm animals, particularly swine, were attributed to contaminated animal feeds (2, 4). Certain domestic animals (cattle, swine, turkey, chicken, ducks) consuming sublethal levels of aflatoxin developed a toxicity syndrome characterized by liver damage (2). Aflatoxins might also have been responsible for liver disease in dogs known as hepatitis-x (82). Monkeys were found to be susceptible to the hepatotoxic effects of aflatoxin and human tissue culture cell lines were lysed at very low levels of toxin (127). No cases of human intoxications have been cited although some indirect evidence has been found (28).

In acute toxicity studies, aflatoxin B_1 has normally been used since it is the most easily purified. LD_{50} values for aflatoxin B_1 range from 0.5 to 10 mg/kg depending on the animal species (130), with older animals more resistant than younger animals (2). The relative toxicities of the aflatoxins to ducklings was determined to be $B_1 > G_1 > B_2 > G_2$. In all species affected, aflatoxin B_1 was the most toxic (130). Purchase (90) found aflatoxin M_1 to be approximately as toxic to ducklings as B_1. Examination of animals following aflatoxin treatment revealed a general pattern of liver damage characterized primarily by

proliferation of the bile duct cells. Other pathological symptoms of aflatoxin poisoning have been summarized by Wogan (130). Zuckerman et al. (135, 136) demonstrated the effects of aflatoxin on tissue cultures of human liver cells and found LD_{50} levels of 1.0 ppm for aflatoxin B_1, 5.0 ppm for G_1, and 16.0 ppm for G_2.

Aflatoxin B_1 also has shown a strong teratogenic effect in hampsters (33). When hampsters were sacrificed on the ninth day of pregnancy following administration of a 4 mg/kg dose of B_1 on the eighth day, 29.4% of the fetuses were malformed and 17.6% were dead.

Feeding of aflatoxins at sublethal levels for extended periods has resulted in the development of hepatomas. Lancaster et al. (62) first noted this with rats in 1961. Newberne (81) has shown that 1.8 ppm aflatoxin B_1 fed to rats for 370 days will produce a 90% incidence of liver tumors. However, Barnes and Butler (11) determined that continual feeding of aflatoxin was not necessary for the initiation of the tumors.

Aflatoxins have been shown to be carcinogenic at sublethal levels to two other species, the rainbow trout and the duck. Epizootics of liver cancer in hatchery-reared rainbow trout were linked to the presence of aflatoxin-contaminated cottonseed meal in the diet (39, 47, 113). With aflatoxin B_1, significant hepatoma incidence occurred at levels as low as 0.5 ppb (46). With ducklings it was demonstrated that 30 ppb aflatoxins fed for 14 months induced liver tumor development (18).

However, some animals are not subject to the toxic effects of aflatoxin. Allcroft (2) found that sheep fed at sublethal levels were not subject to the liver damaging effects of aflatoxin B_1, while other domestic farm animals, including some ruminants, were. Aflatoxin B_1 has also been shown to be relatively ineffective in producing characteristic toxicity (86) and liver cancer symptoms (81) in mice.

Comparisons of aflatoxin metabolism between mice and rats by Portmann et al. (87) precipitated a controversy concerning the nature of the biologically active form of aflatoxin B_1. In their initial study Portmann et al. (87) found that aflatoxin B_1 disappeared more rapidly from rat liver slices than mouse liver slices. Since B_1 in the diet is more carcinogenic to rats than to mice, he suggested that a metabolite of B_1 might be the carcinogenic agent. The metabolism of aflatoxin B_1 proceeds by hydroxylation (30, 55) to derivatives such as aflatoxin M_1. Portmann et al. (87) originally suggested that M_1 might be the active form of aflatoxin. Purchase (90) lent support to this hypothesis by finding that aflatoxin M_1 was as toxic to ducklings as aflatoxin B_1. Roy (99) found that aflatoxin B_1 did not inhibit ribonucleic acid (RNA) polymerase in vitro, while others found this enzyme was inhibited by intraperitoneal injection of B_1 (42). Therefore, Roy (99) postulated that a metabolite was the inhibitor and was responsible for the in vivo inhibition of RNA synthesis that he noted. However, this was not considered conclusive proof, since RNA polymerase need not be

inhibited to obtain an inhibition of RNA synthesis. In fact, Portmann et al. (88) contradicted their own initial observations by finding that the slower disappearance of B_1 in mice was due to a slower transport of B_1 across the cell membranes in mice than in rats. In their kinetic studies, Portmann et al. (88) reported that the hydroxylation reaction actually occurs faster in mice than in rats and on this basis postulated that hydroxylation may be a detoxification mechanism in mice.

From biochemical studies, evidence now points toward messenger RNA synthesis as the site of action by aflatoxin B_1 at toxic levels. Clifford and Rees (24) noted that orotic acid incorporation into RNA and protein synthesis were inhibited by aflatoxin B_1 in an in vitro study with rat liver slices. Synthesis of 45S nucleolar RNA in rats was inhibited by B_1 (41). Roy (99) found an in vivo inhibition of RNA synthesis by feeding aflatoxin B_1 to rats. Further work by Clifford and Rees (25) revealed that orotic acid was incorporated into the nucleotide pool of the nuclei but not into RNA, which suggested that aflatoxin B_1 was interfering with RNA polymerase activity and, thereby, inhibiting messenger RNA synthesis.

The nature of the interference by aflatoxin with RNA polymerase is debatable. Gelboin et al. (42) and Roy (99) suggested that the direct inhibition of the polymerase enzyme may be responsible for the inhibition of RNA synthesis. However, Sporn et al. (117) and Clifford and Rees (24) found that aflatoxin B_1 binds to deoxyribonucleic acid

(DNA). The conclusion from these studies was that B_1 inhibited messenger RNA synthesis by binding to DNA and interfering with the template required by RNA polymerase. These authors postulated that the resulting decreased level of protein synthesis might have been responsible for hepatic necrosis.

However, the binding of aflatoxin B_1 to DNA is not exclusive. Sporn et al. (117) showed that B_1 binds to RNA and Black and Jirgensons (12) demonstrated a binding to histones. Aflatoxin B_1 also has been shown to bind to proteins in bacterial cell membranes (70). Clifford et al, (27) have shown that G_1 and G_2 bind to DNA but to a lesser extent than B_1. The nature of the binding of the aflatoxins to DNA is not known. Clifford and Rees (26) determined that greatest interaction of aflatoxins B_1, G_1, and G_2 occurred with the purine bases. Some workers believe that aflatoxin B_1 functions as an alkylating agent (68, 134). However, Clifford and Rees (26) have shown that B_1 can be separated from DNA on a Sephadex G-50 column, indicating that a weaker bonding is involved.

Further support for DNA binding as the causative factor in aflatoxin toxicity has come from the observation by several workers that DNA synthesis was also inhibited (31, 67, 135, 136). Some workers have attributed this inhibition to a direct effect on DNA polymerase (134), while others suggested that B_1 was bound to DNA and inhibited its primer activity in the polymerase reaction (31).

Aflatoxin B_1 has been shown to affect protein synthesis. Clifford and Rees (24), using toxic levels (7 mg/kg), demonstrated an in vitro inhibition of protein synthesis of 42% in rat liver slices in one hour and pointed out that this could have been due to an inhibition of messenger RNA synthesis. Shank and Wogan (106), feeding a sublethal dose of 600 µg/kg/day of aflatoxin B_1 over a five day period, reported a biphasic response in protein synthesis with an initial inhibition followed by a stimulation. At a level of 60 µg/kg/day for five days, no effect on protein synthesis was noted.

Aflatoxin B_1 markedly affects enzyme activity in the liver. By feeding a toxic level, Brown and Abrams (15) were able to show that the activities of certain mitochondrial dehydrogenases and electron transfer catalysts were decreased. Increases were found in the activities of certain enzymes in the sera of both ducklings and chickens (15) and was postulated to be due to liver lesions. Clifford and Rees (25) found a decrease in hepatic enzymes and a concomitant increase in serum enzymes while feeding at a toxic level (7 mg/kg). Feeding a toxic dose of aflatoxin B_1 (3-5 mg/kg) resulted in an inhibition of the hydrocortisone induction of rat liver tryptophan pyrrolase and tyrosine transaminase (132). The effects of aflatoxin B_1 on RNA polymerase are somewhat contradictory. After intraperitoneal injection of 1 mg of B_1/kg to rats, Gelboin et al. (42) demonstrated a 70% inhibition of RNA polymerase in vivo within two hours. A partial recovery to only 10%

inhibition was found within 24 hours. Roy (99) reported no inhibition by B_1 on an *in vitro* system containing rat testicular RNA polymerase. King and Nicholson (59) reported that the addition of 25-42 μmoles of B_1 to an *in vitro* mixture containing *E. coli* RNA polymerase had no effect on RNA synthesis. They demonstrated that 10 μg/ml of actinomycin D inhibited the polymerase activity by 90% and postulated from this that aflatoxin interacts with DNA rather than the polymerase.

For DNA polymerase a similar controversy exists. Wragg *et al.* (134) showed that the presence of aflatoxin B_1 (5 μg/ml) gave a 60% decrease in DNA produced by *E. coli* and suggested that this was due to an inhibition of DNA polymerase. De Recondo (31) showed that B_1 interfered with DNA and RNA synthesis in rat liver after partial hepatectomy but found no change in the enzymes of DNA synthesis.

No work has been reported on the effects of feeding aflatoxin B_1 at sublethal levels on liver enzyme activities.

EXPERIMENTAL

Experimental Animals

Fish utilized in this study were Mt. Shasta strain rainbow trout (Salmo gairdnerii) which had been artificially spawned and reared at the Food Toxicology and Nutrition Laboratory at this university. Circular fiberglass tanks supplied with well water at a constant temperature of 11° C and a flow rate of about 5 gal./min. were used to rear the fish. The fish were fed ad libitum eight times daily throughout this growth period and the experimental trials.

Enzyme Extraction

The fish were sacrificed with a sharp blow on the head and the livers were immediately removed. After perfusion with modified Kreb's saline (54), the livers were frozen in liquid nitrogen and transported to the laboratory. Eight frozen livers were weighed and a 1:5 (w/v) homogenate was made in 0.01 M Tris-citrate buffer (pH 7.0). Homogenization was performed in a micro container attachment (Eberbach Corp.) to a Waring Blendor for one minute at a rheostat setting of 90. The time between death and homogenization of the livers was usually 30 minutes and never more than one hour. The homogenate was centifuged at 2500 x g for 10 minutes in a Servall Superspeed centrifuge in a cold room (4° C). Four ml of this supernatant was centrifuged at 100,000 x g for 60

minutes in a Beckman Model L-2 refrigerated ultracentrifuge. This supernatant was used for the spectrophotometric enzyme assays and for electrophoresis.

Spectrophotometric Assay Method

Total enzyme activity was determined by measuring the change in absorbance at 340 mμ in a Beckman DB recording spectrophotometer. The change in absorbance was due to the conversion of oxidized cofactor, either nicotine adenine dinucleotide (NAD), or nicotine adenine dinucleotide phosphate (NADP), to reduced cofactor (NADH or NADPH) which absorbs maximally at 340 mμ. For malate dehydrogenase (MDH) and lactate dehydrogenase (LDH), the decrease in absorbance due to the conversion of NADH to NAD was measured. With glucose-6-phosphate dehydrogenase (G6PDH), NADP-isocitrate dehydrogenase (NADP-ICDH), and NAD-isocitrate dehydrogenase (NAD-ICDH), the increase in absorbance was measured as NAD or NADP was converted to NADH or NADPH.

Assay conditions are given in Table 1. These reaction mixtures resulted in maximal enzyme activity at pH 7.5. Sodium pyruvate, sodium glucose-6-phosphate, and NADP were obtained from Sigma Chemicals. All other materials were obtained from Calbiochem.

In each assay, 2.5 ml of the reaction mixture was placed in each of two cuvettes. To the reference cuvette, 0.5 ml of water was added.

Table 1. Spectrophotometric reaction mixtures.

	MDH	LDH	G6PDH	NADP-ICDH	NAD-ICDH
Tris-HCl buffer (M)	0.022	0.026	0.032	0.030	0.024
pH	7.5	7.5	7.5	7.5	7.5
		Substrate (M)			
Oxalacetic acid	8.03×10^{-4}				
Sodium pyruvate		1.6×10^{-3}			
Sodium isocitrate				7.21×10^{-6}	7.21×10^{-6}
Sodium glucose-6-phosphate			1.44×10^{-5}		
		Cofactor (M)			
NADH	2.52×10^{-6}	4.45×10^{-6}			
NAD					2.44×10^{-3}
NADP			3.96×10^{-4}	5.96×10^{-4}	
		Others (M)			
Mn^{+2}				1.6×10^{-2}	1.6×10^{-2}
Mg^{+2}			1.596×10^{-4}		

The reaction was started by adding 0.5 ml of enzyme solution to the other cuvette. The enzyme solution used for G6PDH, NADP-ICDH, NAD-ICDH, and LDH was a 1:50 dilution of the supernatant from the ultracentrifuge, while for MDH, a 1:100 dilution was used. The reaction rate was linear for several minutes but the change in absorbance between 30 and 45 secs. was used to calculate the activity of the enzyme. The activity was calculated according to the following formula:

$$\frac{\frac{\Delta A \times 3}{6.22 \times 10^6} \times \text{dilution factor}}{\text{mg protein/ml}} = \mu\text{moles substrate converted/min/mg}$$

The change in absorbance is multiplied by three since each cuvette contained 3 ml, and 6.22×10^6 is the extinction coefficient of NAD and NADP. The protein concentration was determined by the E_{280}/E_{260} ultraviolet absorption method (22). Assays were performed in duplicate and the results are presented as the mean of these duplicates. Appendix A presents further details on the assay procedures.

Electrophoresis

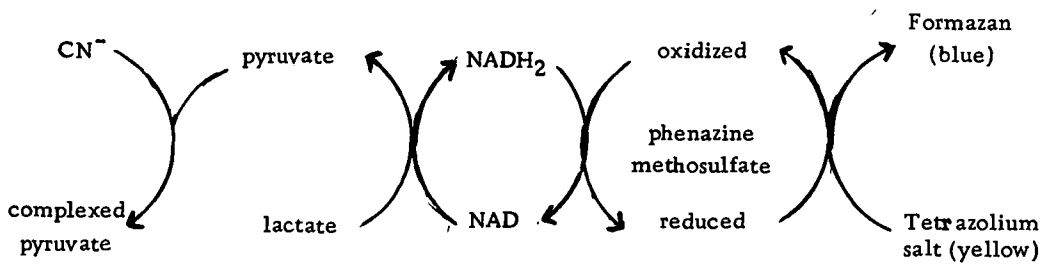
The enzymes were separated by discontinuous polyacrylamide vertical gel electrophoresis in a Model EC-470 electrophoretic cell (E-C Apparatus Corp., Philadelphia, Pa.). The discontinuous method is described in detail in E-C bulletin, Vol. II., No. 1 (36) and by Petropakis (84).

The spacer gel buffer (0.062 M Tris-HCl, pH 6.7) was prepared by dissolving 22.5 gm. of Tris and 12 ml of concentrated HCl in water to a final volume of three liters. Three liters of running gel buffer (0.38 M Tris-HCl, pH 9.0) was prepared by dissolving 138 gm. of Tris and 12 ml of concentrated HCl in water. Electrode buffer (0.0165 M Tris-0.0390 M glycine, pH 8.75) was prepared by dissolving 11.6 gm. glycine and 8.0 gm. of Tris in four liters of water. Plug and running gel solutions were prepared by dissolving appropriate amounts of Cyanogum-41 (95% acrylamide and 5% N, N'-methylenebisacrylamide, E-C Apparatus Corp.) in running gel buffer to make 100 ml of 10% and 7% concentrations, respectively. Spacer gel solution was made by dissolving 4 gm. of Cyanogum-41 in 100 ml of spacer gel buffer to yield a 4% solution. To each of these 100 ml solutions, 0.1 ml of TMED (N, N, N', N'-tetramethylethylene diamine) and 0.02 ml of Tween 80, a wetting agent, were added. The solutions were filtered through Whatman No. 12 filter paper. Since these solutions were unstable, they had to be prepared immediately prior to use (97). To effect polymerization of the gel solutions, 0.1 gm. of ammonium persulfate was added.

The assembly and operation of the electrophoretic cell for the discontinuous method are described in detail (35, 36, 84), and will not be discussed here. The conditions used in this study were identical to those of Petropakis (84). The length of the electrophoretic run was

usually 3 1/2 hours. Upon completion of the run, the gel was cut into three slices and each slice was stained for activity in the incubating medium described in Table 2. The incubation time in the dark at 37° C for LDH and MDH was 25 minutes and for G6PDH was either 45 minutes or 4 hours, depending on the particular run. Bulk lots of the incubating solutions were made. These solutions contained the substrate and cofactors for the reaction and were stable up to four days at refrigeration temperatures. The use of these bulk solutions appeared to increase the reproducibility of the results. Since nitro blue tetrazolium and phenazine methosulfate are quite light sensitive, these substances were added to the solutions immediately prior to incubation. Photographs were taken of the isozyme patterns to serve as a permanent record.

The staining reactions were given by Wilkinson (126) and the overall reaction for LDH was as follows:



The reactions for the other enzymes were similar to this.

Experimental Diets

Four different diets were fed in the feeding trials conducted in this study. The formula for the basic control diet (Diet #1) is given in

Table 2. Electrophoretic incubating media.

	LDH	MDH	G6PDH
Total volume	92 ml	100 ml	100 ml
Buffer	0.05 M Tris-HCl	0.05 M Tris-HCl	0.05 M Tris-HCl
pH	7.5	7.5	7.5
	Substrates		
sodium lactate	1 ml		
malic acid		0.495 gms	
sodium glucose-6-phosphate			0.0826 gms
	Cofactors		
NAD	0.040 gms	0.040 gms	
NADP			0.0028 gms
	Others		
Mg^{+2}			0.0050 gms
nitro blue tetrazolium*	0.042 gms	0.042 gms	0.0076 gms
phenazine methosulfate	0.003 gms	0.003 gms	0.003 gms
KCN (0.06 M)	7.5 ml	7.5 ml	
NaOH (1.0 N)		8.0 ml	

*2, 2'-Di-p-nitrophenyl-5, 5'-diphenyl-3, 3'-(3, 3'-dimethoxy-4, 4'-diphenylene)di-tetrazolium chloride.

Tables 3, 4, and 5. The other diets consisted of the control diet plus other constituents as follows: Diet #61 (100 ppm methyl sterculate), Diet #61-A (200 ppm methyl sterculate), and Diet #30 (20 ppb aflatoxin B_1).

In Experiment 1, 100 eighteen-week-old fish were fed Diet #30. Samples of eight fish each were taken every third day for 15 days and every seventh day thereafter up to the 35th day of feeding. These samples were used for determination of the enzymatic activities. Samples of three fish each were taken at odd intervals for histological examination.

In Experiment 2, 100 fish were fed a diet containing 100 ppm methyl sterculate (Diet #61) for a period of two weeks. Samples of eight fish each were taken every third day throughout this period for enzyme activity studies. After the completion of the two week period, the fish were returned to the control diet, and samples were taken daily for a five-day period. Again, histological samples were taken occasionally throughout the experiment. The fish for this study were the same age as those of Experiment 1.

Experiment 3 varied only slightly from Experiment 2. Diet #61 was fed to 150 fish for a period of 38 days. The initial age of these fish was 21 weeks and the population was sampled daily for 10 days and at odd intervals thereafter. Samples for histological evaluation were taken also.

Table 3. Composition of control diet.

Ingredient	%
Casein (NBC Vitamin-free)	49.5
Gelatin	8.7
Dextrin*	15.6
Mineral Mix**	4.0
Carboxyl Methyl Cellulose***	1.3
alpha-cellulose (Alphacel-NBC)	7.7
Vitamin E (supplies 660 IU/Kg)	0.2
70% Choline Chloride	1.0
Vitamin Mix No. 3	2.0
Salmon Oil	10.0

*American Maize Products Co., 1818 W. Lake Ave. North, Seattle, Washington.

**Bernhart-Tomerelli Salt Mix - Modified by addition of NaF and $CoCl_2$ at 0.002 and 0.020%, respectively.

***Hercules Powder Co., 120 Montgomery St., San Francisco, California.

Table 4. Modified Bernhart-Tomarelli salt mix.

Ingredient	%
$CaCO_3$	2.100
$Ca(PO_4)_2$	73.500
Citric Acid	0.205
Cupric Citrate ($2Cu_2C_6H_5O_7 \cdot 5H_2O$)	0.046
Ferric Citrate ($FeC_6H_5O_7 \cdot 5H_2O$)	0.558
MgO	2.500
$Mn_3(C_6H_5O_7)_2$	0.835
KI	0.001
K_2HPO_4	8.100
K_2SO_4	6.800
NaCl	3.060
$Na_2HPO_4 \cdot 2H_2O$	2.140
$Zn_3(C_6H_5O_7)_2 \cdot 2H_2O$	0.133
NaF	0.002
$CoCl_2$	0.020

Table 5. Vitamin Premix #3.

	% in Mix	% in diet with 2% Premix
Thiamine (HCl)	0.3200	0.0064
Riboflavin	0.7200	0.0144
Niacinamide	2.5600	0.0512
Biotin	0.0080	0.00016
Ca-pantothenate (D)	1.4400	0.0288
Pyridoxine (HCl)	0.2400	0.0048
Folic Acid	0.0960	0.00192
Menadione	0.0800	0.0016
B_{12} (cobalamine - 3,000 μgm/gm)	0.2667	0.005334
i-inositol (meso)	12.5000	0.2500
Ascorbic Acid	6.0000	0.1200
Para-amino-benzoic Acid	2.0000	0.0400
Vitamin D_2 (500,000 usp/g)	0.0400	0.0008
Butylated hydroxy anisole	0.0750	0.0015
Butylated hydroxy toluene	0.0750	0.0015
Vitamin A (250,000 IU/g)	1.0000	50,000 IU/Kg
Celite	72.5793	
	100.0000	

Experiment 4 consisted of placing 80 twenty-three-week-old fish on Diet #61-A (200 ppm methyl sterculate) for a period of 26 days. Samples of fish for enzyme analysis were taken daily for a six-day period and at odd intervals thereafter. Histological samples were taken at odd intervals throughout the trial.

RESULTS

Preparation of Enzyme Extract

As indicated in Table 6, the preparation of the enzyme extract by ultracentrifugation at 100,000 x g resulted in a several-fold purification of LDH, MDH, NADP-ICDH, and G6PDH. This extraction technique was not particularly good for NAD-ICDH since 75% of the total activity was lost during ultracentrifugation. However, complete activity of the other enzymes was retained by this extraction procedure and hence it was used throughout this study.

Activity Studies

In Experiment 1, eighteen-week-old rainbow trout were fed aflatoxin B_1 at a level of 20 ppb in the diet for a 35-day period. Liver enzyme activities were determined from samples of eight fish each at intervals throughout this period. Samples were also taken from the control diet at these same intervals. The results of this feeding trial are shown in Table 7 and Figures 2-6. Although these results showed considerable variation, there was a tendency for the specific activity of G6PDH to increase above the controls toward the end of the feeding trial (Figure 2). In contrast to this, the specific activity of NAD-ICDH was decreased after day 13 to zero on day 20 through 28, followed by a tendency to return to normal levels (Figure 4). The specific activities

Table 6. Purification of dehydrogenases of rainbow trout liver by ultracentrifugation.

	Total units (μmoles / min / ml)	Total protein (mg / ml)	Specific activity (μmoles / min / ml)	Yield (%)	Purification (fold)
		G6PDH			
2,500 x g supernatant	16.9	174	0.097		
100,000 x g supernatant	16.9	46	0.368	100	3.8
		NADP-ICDH			
2,500 x g supernatant	19.3	174	0.111		
100,000 x g supernatant	16.9	46	0.368	88	3.3
		NAD-ICDH			
2,500 x g supernatant	9.7	174	0.056		
100,000 x g supernatant	2.4	46	0.052	25	0.9
		LDH			
2,500 x g supernatant	164	174	0.94		
100,000 x g supernatant	184	46	4.00	112	4.3
		MDH			
2,500 x g supernatant	1250	174	7.2		
100,000 x g supernatant	1250	46	27.3	100	3.8

Table 7. Effect of feeding aflatoxin B_1 (20 ppb) on certain rainbow trout liver enzymes.

Days fed	Protein (mg/ml)	G6PDH		NADP-ICDH		NAD-ICDH		LDH		MDH	
		Total[1] activity	Specific[2] activity	Total activity	Specific activity	Total activity	Specific activity	Total activity	Specific activity	Total activity	Specific activity
0	53.0	16.9	0.32	26.5	0.50	2.4	0.045	202	3.82	1250	23.6
	(56.5)[3]	(21.7)	(0.38)	(29.0)	(0.51)	(2.4)	(0.043)	(184)	(3.25)	(1350)	(23.9)
3	56.0	24.0	0.43	29.0	0.52	2.4	0.043	198	3.54	1350	24.1
	(49.0)	(21.7)	(0.44)	(29.0)	(0.59)	(2.4)	(0.049)	(180)	(3.66)	(1450)	(29.6)
6	57.5	21.7	0.38	24.0	0.42	2.4	0.042	193	3.36	1640	28.5
	(50.5)	(17.0)	(0.34)	(21.7)	(0.43)	(2.4)	(0.048)	(174)	(3.44)	(1160)	(22.9)
13	52.0	21.7	0.42	24.0	0.46	2.4	0.046	169	3.25	1160	22.3
	(44.0)	(21.7)	(0.44)	(24.0)	(0.55)	(2.4)	(0.055)	(164)	(3.74)	(1160)	(26.4)
20	41.0	14.5	0.35	14.5	0.35	0	0	158	3.84	965	23.5
	(52.0)	(21.7)	(0.34)	(20.5)	(0.40)	(1.2)	(0.023)	(178)	(3.44)	(1160)	(22.3)
28	51.0	29.0	0.57	21.7	0.43	0	0	186	3.64	1350	26.4
	(44.5)	(12.1)	(0.27)	(19.3)	(0.43)	(1.2)	(0.027)	(198)	(4.44)	(1350)	(30.3)
30	46.0	19.3	0.42	19.3	0.42	0.6	0.013	164	3.59	1250	27.4
	(50.0)	(19.3)	(0.39)	(21.7)	(0.43)	(1.8)	(0.036)	(181)	(3.61)	(1250)	(24.5)
35	49.0	24.0	0.49	19.3	0.39	1.0	0.019	180	3.68	1200	24.6
	(44.0)	(18.1)	(0.41)	(16.9)	(0.38)	(2.0)	(0.045)	(154)	(3.50)	(1060)	(24.0)

[1] Total activity values are expressed as μmoles substrate converted per minute per ml of extract.

[2] Specific activity values are expressed as μmoles substrate converted per minute per mg of protein.

[3] Values in parentheses are for paired control samples fed on Diet #1 and assayed on the same day as the experimental diet.

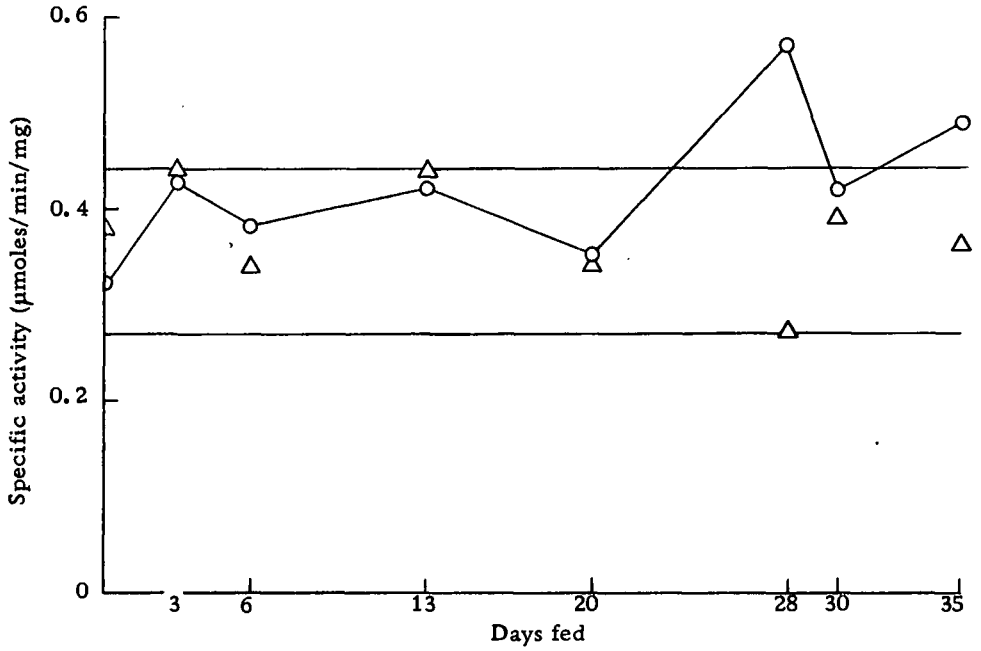


Figure 2. Effect of feeding aflatoxin B_1 at 20 ppb on G6PDH. Triangles are control values. Circles are values for Diet #30 (20 ppb of aflatoxin B_1). Horizontal lines represent upper and lower limits of the control.

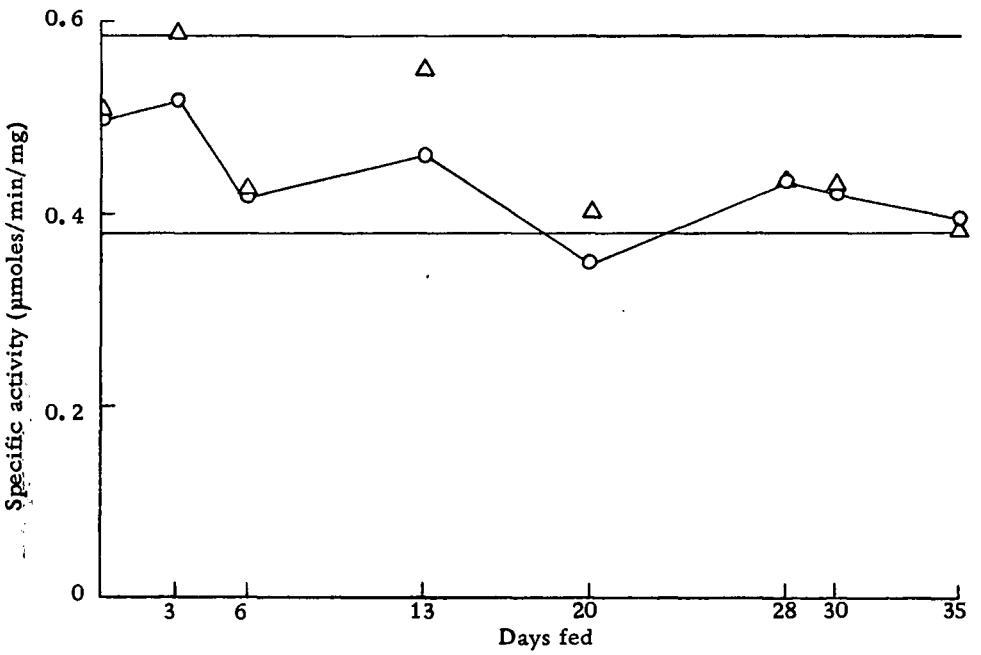


Figure 3. Effect of feeding aflatoxin B_1 at 20 ppb on NADP-ICDH. Triangles are control values. Circles are values for Diet #30 (20 ppb aflatoxin B_1). Horizontal lines represent upper and lower limits of the control.

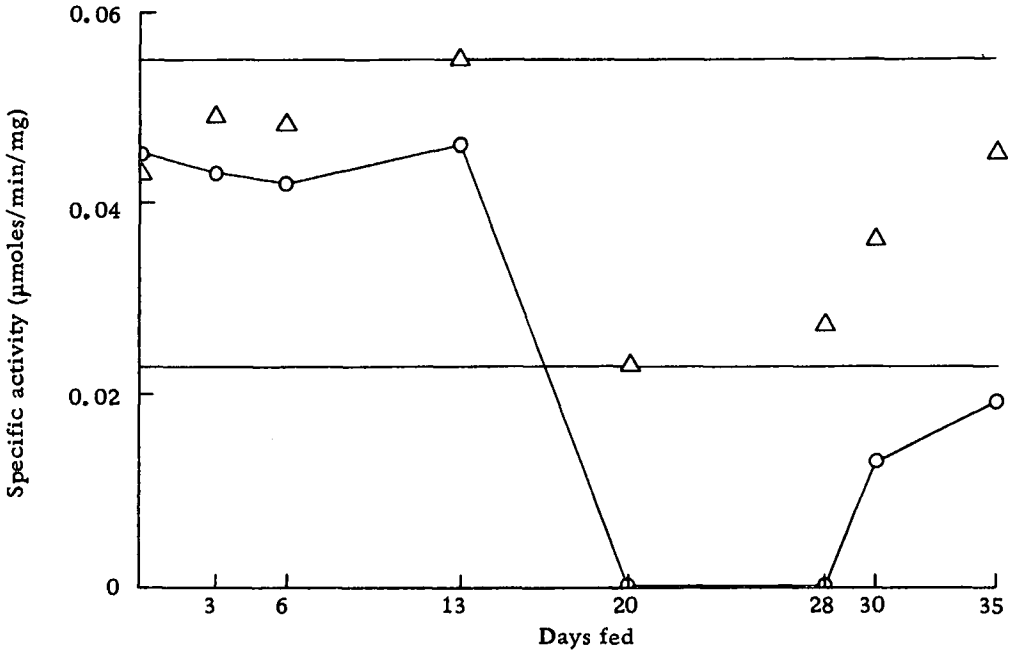


Figure 4. Effect of feeding aflatoxin B_1 at 20 ppb on NAD-ICDH. Triangles are control values. Circles are values for Diet #30 (20 ppb aflatoxin B_1). Horizontal lines represent upper and lower limits of the control.

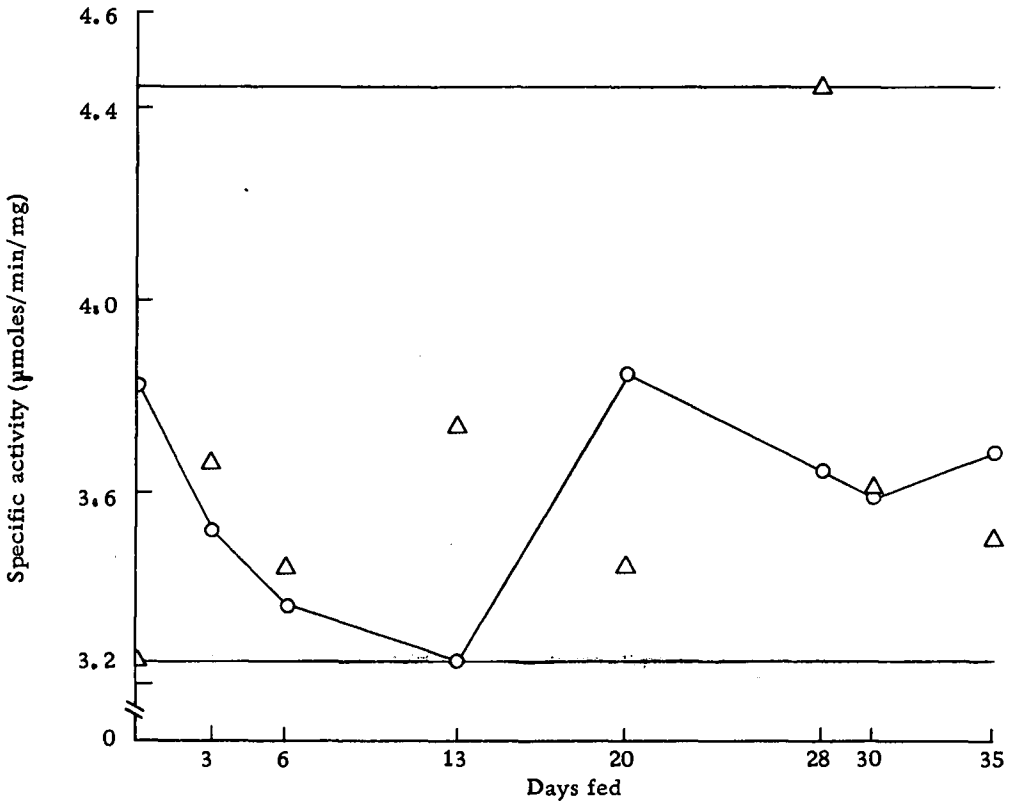


Figure 5. Effect of feeding aflatoxin B_1 at 20 ppb on LDH. Triangles are control values. Circles are values for Diet #30 (20 ppb aflatoxin B_1). Horizontal lines represent upper and lower limits of the control.

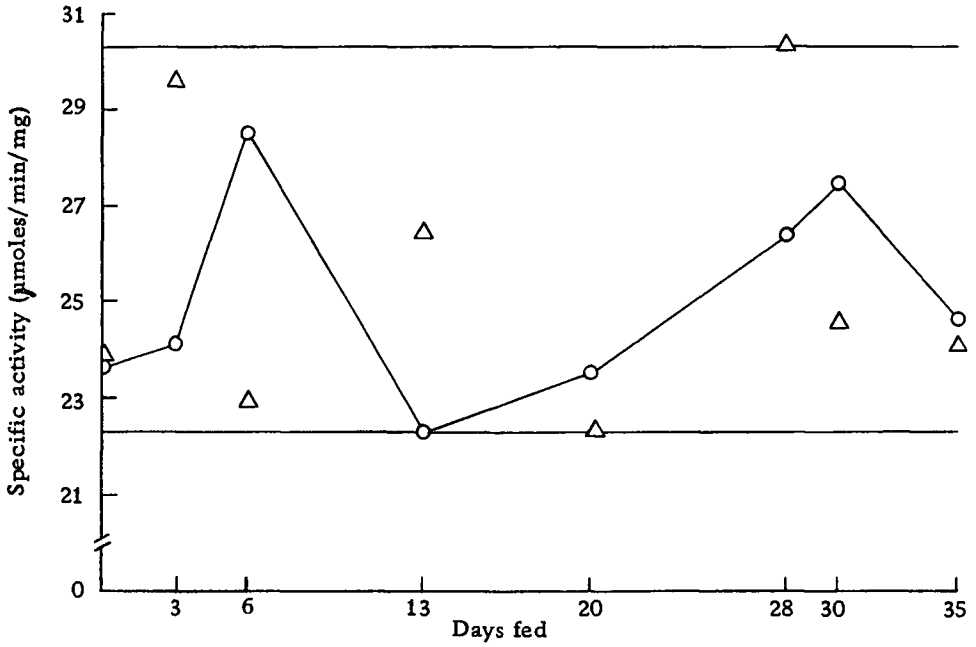


Figure 6. Effect of feeding aflatoxin B_1 at 20 ppb on MDH. Triangles are control values. Circles are values for Diet #30 (20 ppb aflatoxin B_1). Horizontal lines represent upper and lower limits of the control.

of the other enzymes appeared to maintain their normal levels throughout the feeding period.

Eighteen-week-old rainbow trout were fed sterculate at 100 ppm in the diet for 14 days in Experiment 2. At the completion of this two-week period, the trout were returned to the control diet for one week. Enzyme activities and protein were determined at intervals throughout this three week period. Trout on the control diet were also sampled during this feeding trial. The results, presented in Table 8 and Figures 7-11, reveal a noticeable decrease in the specific activities of G6PDH, LDH and MDH below the control range after three days. After six days the specific activities of these enzymes returned to the normal range for the remainder of the 14-day period. The specific activity of NADP-ICDH did not vary markedly from the control range. With NAD-ICDH some initial decrease in specific activity was noted; however, this decrease did not fall below the lowest control value, which happened to be much lower than any of the other control values. If this lowest value were deleted as being abnormal, then the decrease in specific activity of NAD-ICDH would have been quite noticeable. In general, there was an increase in specific activity, followed by a decrease on day 20, after switching this group of trout to the control diet. Although both the protein content and the total activities of all the dehydrogenases showed a decrease (Table 8), the fluctuation in the specific activity was caused by a more rapid decrease in the protein

Table 8. Effect of feeding methyl sterculate (100 ppm) for 14 days and then control diet on certain liver enzymes of 18-week-old rainbow trout.

	Days fed	Protein (mg/ml)	G6PDH		NADP-ICDH		NAD-ICDH		LDH		MDH	
			Total[1] activity	Specific[2] activity	Total activity	Specific activity	Total activity	Specific activity	Total activity	Specific activity	Total activity	Specific activity
Sterculate	0	52.5	21.7	0.41	29.0	0.55	1.9	0.036	212	4.03	1350	25.7
		(56.5)[3]	(21.7)	(0.38)	(29.0)	(0.51)	(2.4)	(0.043)	(184)	(3.25)	(1350)	(23.9)
	3	62.0	16.9	0.27	21.7	0.35	1.5	0.024	145	2.34	960	15.5
		(49.0)	(21.7)	(0.44)	(29.0)	(0.59)	(2.4)	(0.049)	(180)	(3.66)	(1450)	(29.6)
	6	54.0	19.5	0.36	24.0	0.44	1.2	0.022	179	3.31	1350	25.0
		(50.5)	(17.0)	(0.34)	(21.7)	(0.43)	(2.4)	(0.048)	(174)	(3.44)	(1160)	(22.9)
	9	46.0	16.9	0.37	19.3	0.42	2.4	0.052	164	3.57	1250	27.2
		(61.0)	(21.7)	(0.35)	(24.0)	(0.39)	(2.4)	(0.039)	(202)	(3.32)	(1350)	(22.1)
	13	52.0	16.9	0.33	19.3	0.37	2.4	0.046	164	3.16	1160	22.3
		(44.0)	(21.7)	(0.44)	(24.0)	(0.55)	(2.4)	(0.055)	(164)	(3.74)	(1160)	(26.4)
Control	0	52.0	16.9	0.33	19.3	0.37	2.4	0.046	164	3.16	1160	22.3
		(44.0)	(21.7)	(0.44)	(24.0)	(0.55)	(2.4)	(0.055)	(164)	(3.74)	(1160)	(26.4)
	1	44.0	14.5	0.33	19.3	0.44	2.4	0.055	154	3.51	960	21.9
	2	41.0	14.5	0.35	16.9	0.41	2.4	0.059	154	3.77	1060	25.8
	3	36.5	14.5	0.40	14.5	0.40	1.8	0.044	111	3.04	770	21.2
		(44.0)	(19.3)	(0.44)	(24.0)	(0.55)	(2.4)	(0.055)	(164)	(3.74)	(1160)	(26.4)
	5	36.0	16.9	0.47	14.5	0.40	2.4	0.067	108	3.01	870	24.1
	7	41.0	9.7	0.24	12.1	0.29	0.6	0.015	96	2.35	770	18.6
		(52.0)	(21.7)	(0.34)	(20.5)	(0.40)	(1.2)	(0.023)	(178)	(3.44)	(1160)	(22.3)

[1] Total activity values are expressed as μmoles substrate converted per minute per ml of extract.

[2] Specific activity values are expressed as μmoles substrate converted per minute per mg of protein.

[3] Values in parentheses are for paired control samples fed on Diet #1 and assayed on the same day as the experimental diet.

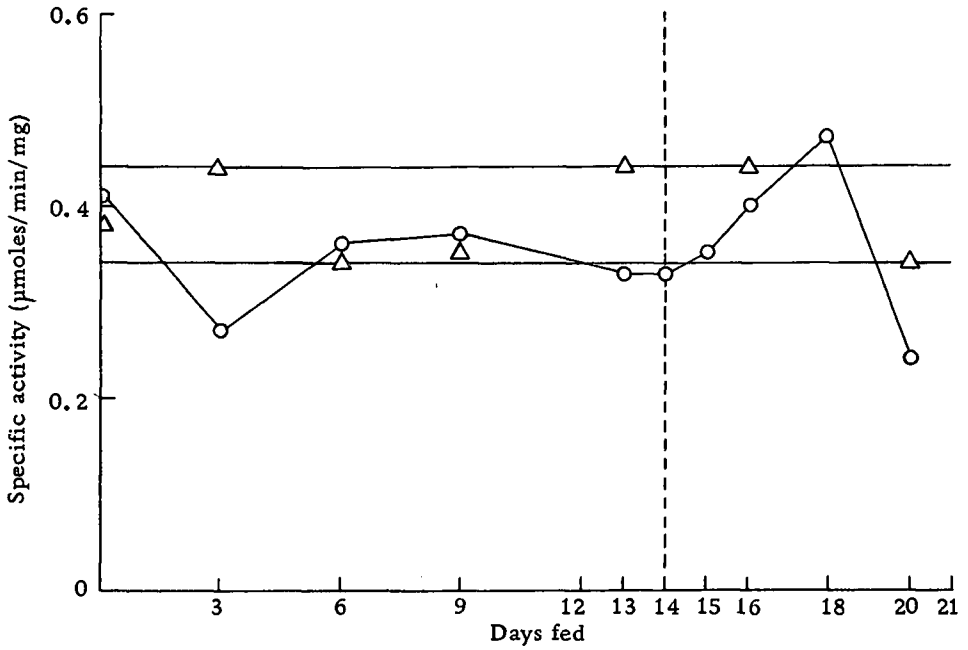


Figure 7. Effect of feeding sterculate at 100 ppm on G6PDH of 18-week-old fish. Triangles are control values. Circles are values for Diet #61 (100 ppm sterculate). Horizontal lines represent upper and lower limits of the controls. The dotted line shows the point where fish were returned to control diet.

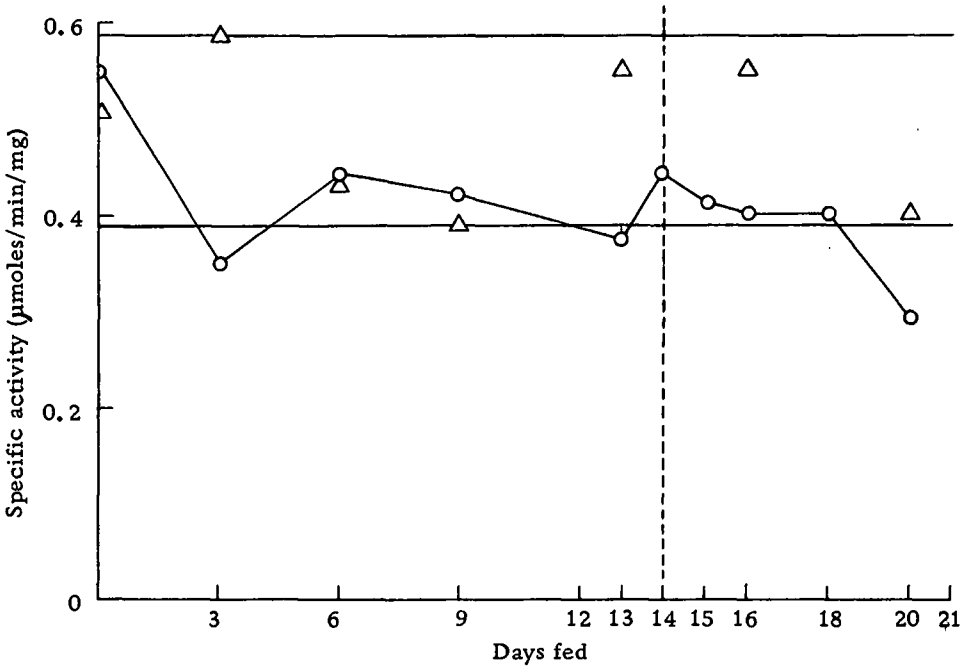


Figure 8. Effect of feeding sterculate at 100 ppm on NADP-ICDH of 18-week-old fish. Triangles are control values. Circles are values for Diet #61 (100 ppm sterculate). Horizontal lines represent upper and lower limits of the controls. The dotted line shows the point where the fish were returned to control diet.

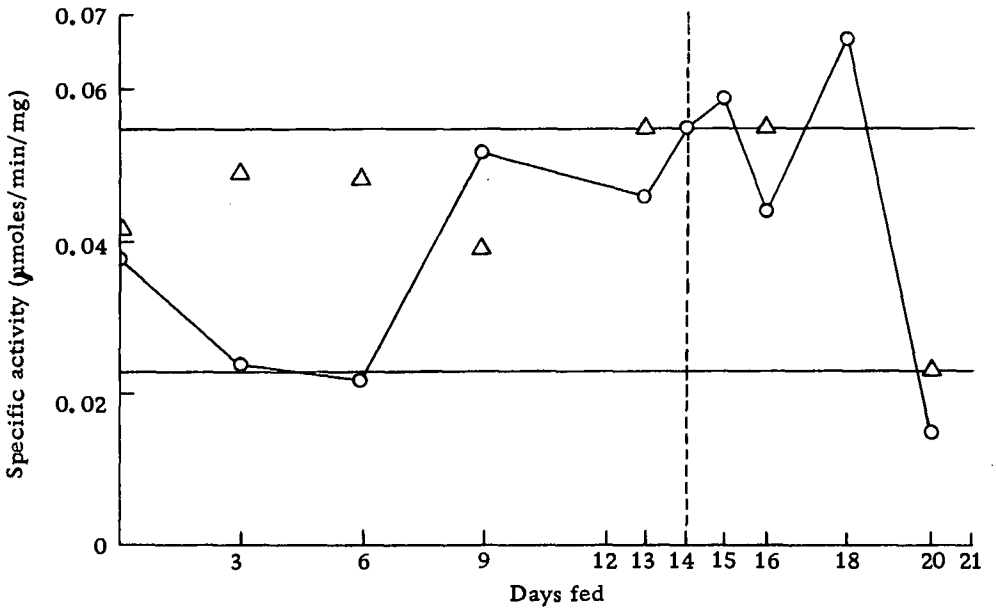


Figure 9. Effect of feeding sterculate at 100 ppm on NAD-ICDH of 18-week-old fish. Triangles are control values. Circles are values for Diet #61 (100 ppm sterculate). Horizontal lines represent upper and lower limits of the control. The dotted line shows the point where fish were returned to control diet.

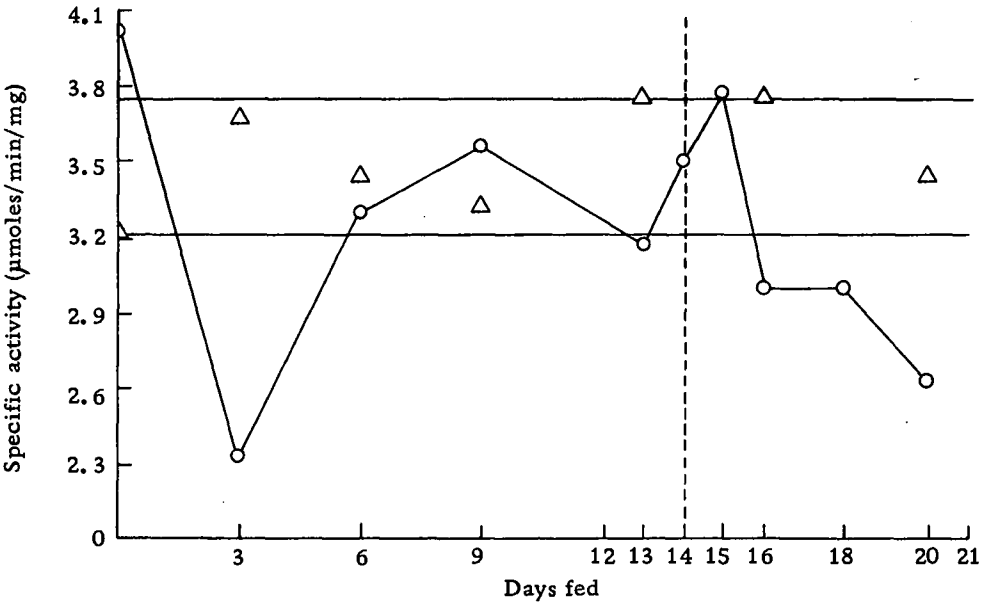


Figure 10. Effect of feeding sterculate at 100 ppm on LDH of 18-week-old fish. Triangles are control values. Circles are values for Diet #61 (100 ppm sterculate). Horizontal lines represent upper and lower limits of the control. The dotted line shows where the fish were returned to control diet.

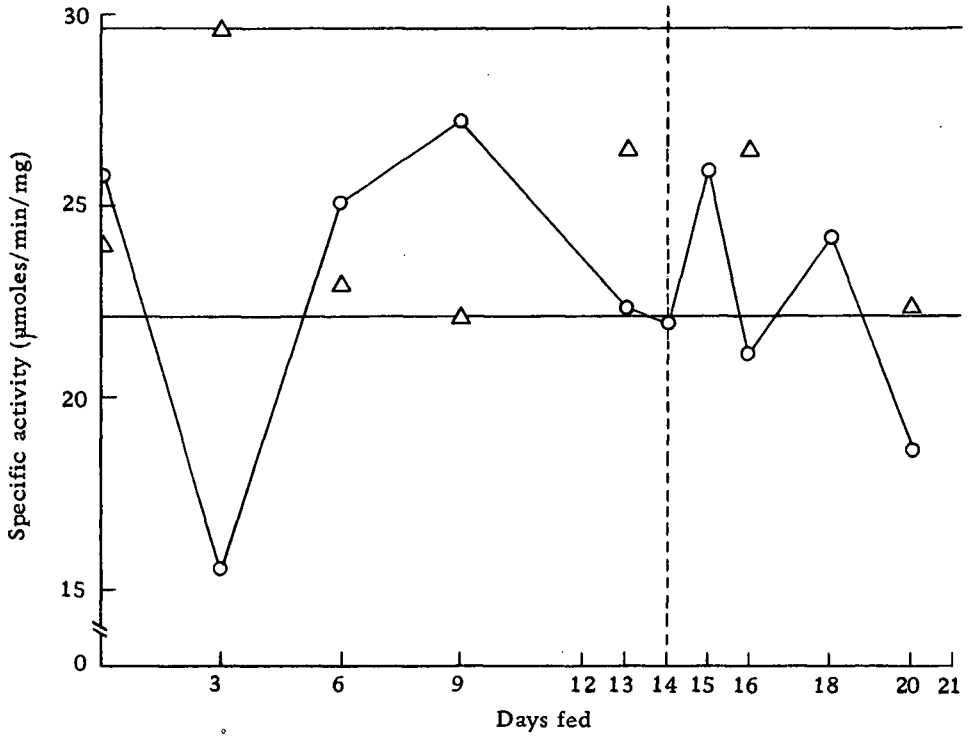


Figure 11. Effect of feeding sterculate at 100 ppm MDH of 18-week-old fish. Triangles are control values for Diet #61 (100 ppm sterculate). Horizontal lines represent upper and lower limits of the control. The dotted line shows the point where fish were returned to control diet.

level than the dehydrogenase activities. An insufficient number of fish was used in this experiment to determine how long the dehydrogenase activities would remain at this depressed level.

In an attempt to repeat Experiment 2, sterculate was fed at a level of 100 ppm to 21-week-old rainbow trout for 38 days in Experiment 3. In this case, liver enzymes were assayed every day for nine days and at odd intervals thereafter. The livers of the 21-week-old fish were approximately twice as large as those of the 18-week-old trout used in Experiment 2. Consequently, the effects of sterculate fed at 100 ppm on these fish, as documented in Table 9 and Figures 12-16, were quite different from the effects seen in Experiment 2. Table 9 shows a general decrease in both total and specific activities for all the enzymes studied throughout the 38-day feeding period with little, if any, indication of a return to normal total activity levels. Protein levels in the liver were lower than normal from day 24 through day 28. G6PDH specific activity was below the lower control limit throughout the feeding period (Figure 12). However, the decrease in specific activity did not become markedly variant from the controls until the fifth day of feeding. NADP-ICDH specific activity decreased after one day of feeding (Figure 13). The specific activity returned within the control range only to be lowered again on the sixth day of feeding. The specific activity again returned to normal but was markedly decreased after 16 days of feeding. This lowered specific activity remained

Table 9. Effect of feeding methyl sterculate (100 ppm) on certain liver enzymes of 21-week-old rainbow trout.

Days fed	Protein (mg/ml)	G6PDH		NADP-ICDH		NAD-ICDH		LDH		MDH	
		Total[1] activity	Specific[2] activity	Total activity	Specific activity	Total activity	Specific activity	Total activity	Specific activity	Total activity	Specific activity
1	47.5	12.1	0.24	14.5	0.31	1.0	0.020	212	4.48	1350	28.5
	(51.0)[3]	(22.6)	(0.44)	(25.3)	(0.50)	(3.0)	(0.059)	(181)	(3.56)	(1350)	(26.6)
2	46.0	14.5	0.31	16.9	0.37	1.2	0.026	206	4.47	1250	27.2
3	46.5	12.1	0.26	16.9	0.36	1.8	0.039	193	4.15	1350	29.1
4	47.5	14.5	0.30	21.7	0.46	1.2	0.025	240	5.05	1450	30.4
	(48.0)	(22.6)	(0.47)	(25.3)	(0.53)	(2.4)	(0.050)	(217)	(4.52)	(1250)	(26.2)
5	46.0	12.1	0.26	16.9	0.37	1.2	0.026	193	4.20	1060	23.1
6	48.0	9.7	0.20	14.5	0.30	--	--	184	3.82	1010	21.1
7	49.0	12.1	0.25	16.9	0.34	0.6	0.012	208	4.23	1350	27.5
	(45.0)	(15.7)	(0.35)	(19.3)	(0.43)	(1.2)	(0.027)	(193)	(4.29)	(1350)	(30.0)
8	44.5	12.1	0.27	19.3	0.43	1.2	0.027	198	4.44	1350	30.3
9	48.0	12.1	0.25	19.3	0.40	1.2	0.025	208	4.35	1450	30.4
	(51.0)	(15.7)	(0.31)	(21.7)	(0.43)	(1.2)	(0.024)	(193)	(3.78)	(1450)	(28.5)
17	44.0	9.7	0.22	12.1	0.27	2.4	0.055	126	2.85	965	21.9
	(54.0)	(19.3)	(0.36)	(19.3)	(0.36)	(2.4)	(0.045)	(152)	(2.82)	(1160)	(21.4)
20	44.0	9.7	0.22	14.5	0.33	2.4	0.055	130	2.96	820	18.8
22	44.0	9.7	0.22	14.5	0.33	1.0	0.022	130	2.96	675	15.3
	(44.0)	(21.7)	(0.49)	(21.7)	(0.49)	(2.0)	(0.045)	(174)	(3.92)	(1060)	(24.0)
24	32.5	7.3	0.22	7.3	0.22	--	--	116	3.55	770	23.7
28	36.5	9.7	0.26	9.7	0.26	1.2	0.033	106	2.91	580	15.9
	(46.0)	(16.9)	(0.37)	(16.9)	(0.37)	--	--	(184)	(4.00)	(1250)	(27.3)
38	49.0	9.7	0.19	14.5	0.30	1.2	0.024	154	3.15	580	11.8
	(44.0)	(16.9)	(0.38)	(19.3)	(0.44)	(2.4)	(0.055)	(164)	(3.74)	(1060)	(24.0)

[1]Total activity values are expressed as μmoles substrate converted per minute per ml of extract.

[2]Specific activity values are expressed as μmoles substrate converted per minute per mg of protein.

[3]Values in parentheses are for paired control samples fed on Diet #1 and assayed on the same day as the experimental diet.

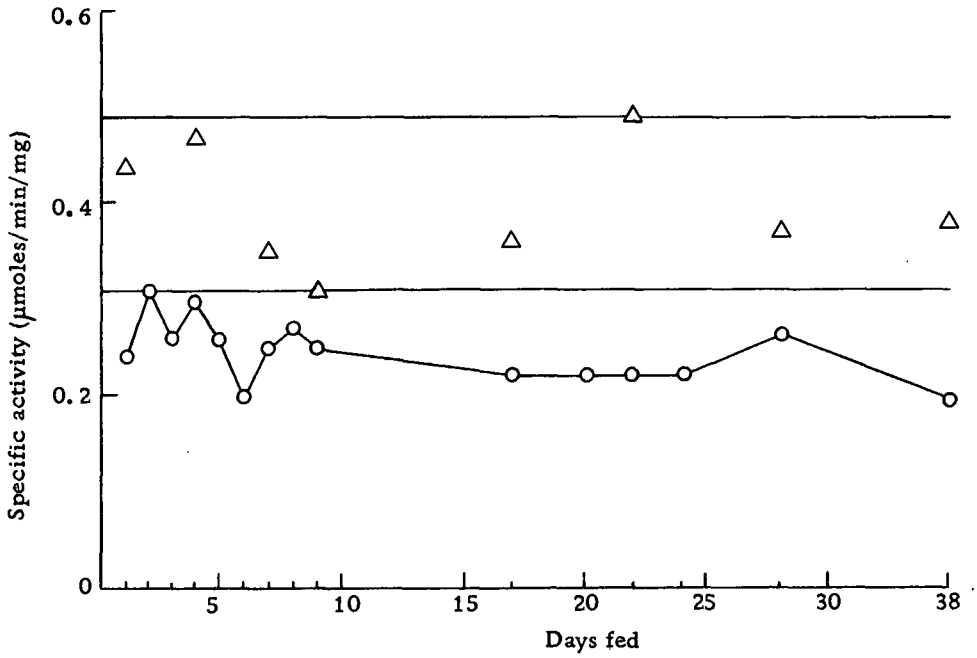


Figure 12. Effect of feeding sterculate at 100 ppm on G6PDH of 21-week-old fish. Triangles are control values. Circles are values for Diet #61 (100 ppm sterculate). Horizontal lines represent upper and lower limits of the control.

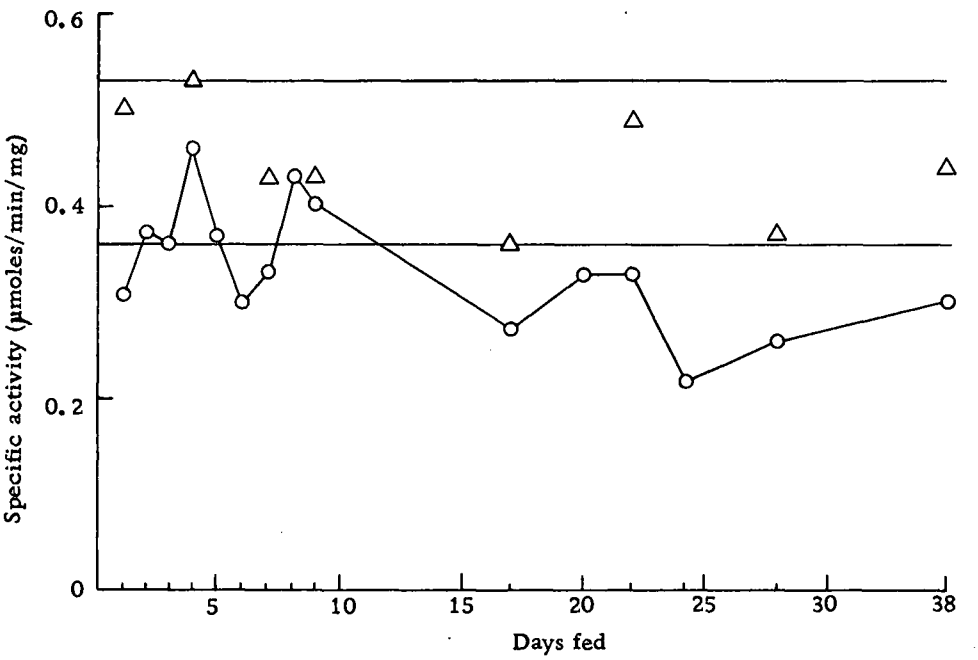


Figure 13. Effect of feeding sterculate at 100 ppm NADP-ICDH of 21-week-old fish. Triangles are control values. Circles are values for Diet #61 (100 ppm sterculate). Horizontal lines represent upper and lower limits of the control.

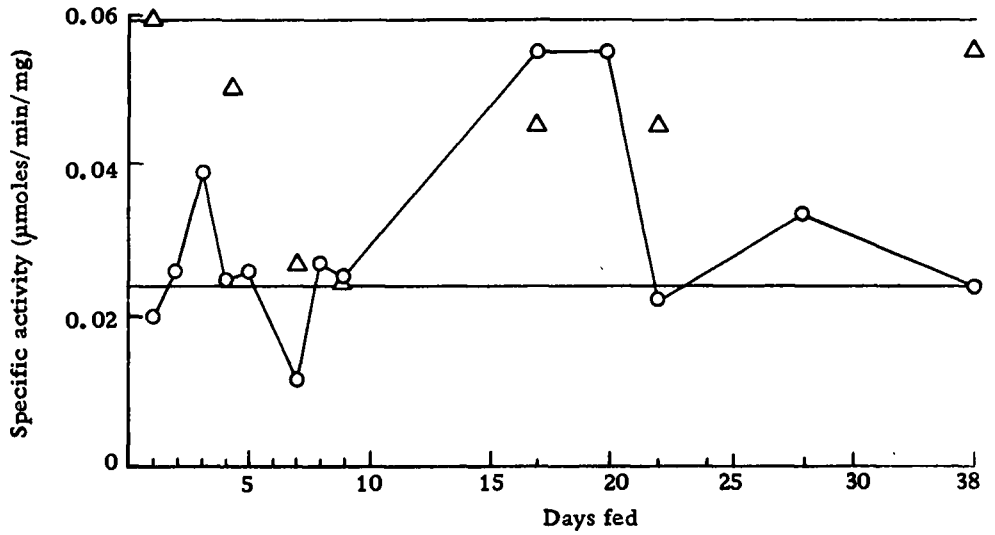


Figure 14. Effect of feeding sterculate at 100 ppm on NAD-ICDH of 21-week-old fish. Triangles are control values. Circles are values for Diet #61 (100 ppm sterculate). Horizontal lines represent upper and lower limits of the control.

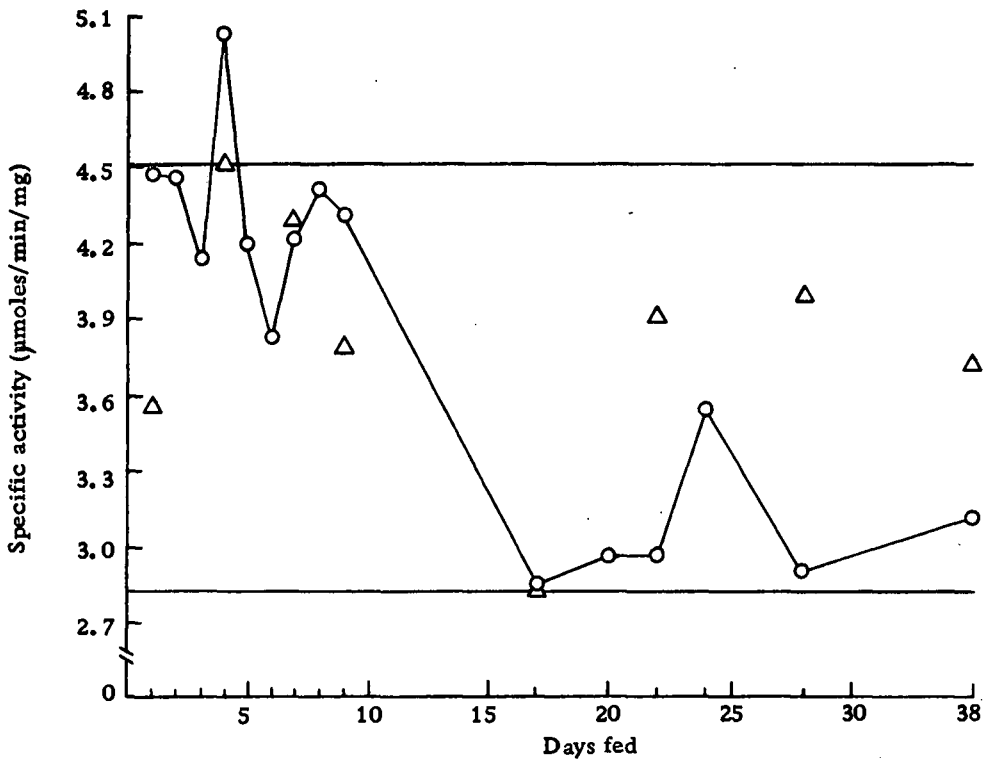


Figure 15. Effect of feeding sterculate at 100 ppm on LDH of 21-week-old fish. Triangles are control values. Circles are values for Diet #61 (100 ppm sterculate). Horizontal lines represent upper and lower limits of the control.

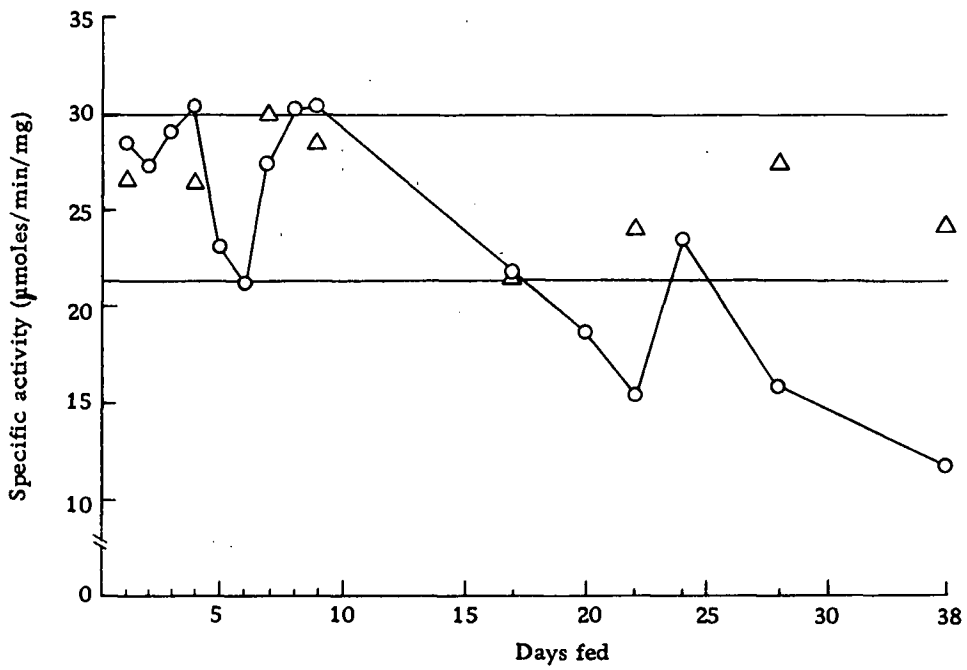


Figure 16. Effect of feeding sterculate at 100 ppm on MDH of 21-week-old fish. Triangles are control values. Circles are values for Diet #61 (100 ppm sterculate). Horizontal lines represent upper and lower limits of the control.

through the remainder of the feeding period. Specific activity of NAD-ICDH fell below the lower control value on the seventh day of feeding (Figure 14). The specific activity returned to normal on the eighth day and remained within the control range through the remainder of the feeding period. With LDH (Figure 15), the results were confused by a low control value. One control value of 2.8 μmoles/min/mg was found, while the next lowest control value was 3.6 μmoles/min/mg. A decrease in LDH specific activity was seen after 17 days of feeding but never fell below this lowest control value. Specific activity of MDH dropped markedly below the controls after 22 days of feeding (Figure 16). No indication was seen that the specific activities of these enzymes would eventually return to normal levels.

In Experiment 4, 23-week-old rainbow trout were fed 200 ppm sterculate in the diet for a period of 26 days. Samples of eight trout livers each were taken for enzyme assays and protein determination from this group and from the control group at intervals throughout the feeding period. The results of this feeding trial are given in Table 10 and Figures 17-21. Table 10 shows a decline in total and specific activities for all five dehydrogenases. The number of days necessary to effect the decline varried depending on the enzyme. The protein level was below normal from day 16 through the end of the feeding trial. An indication of a return to normal activity levels was found on day 22 but an insufficient number of fish prevented the feeding

Table 10. Effect of feeding methyl sterculate (200 ppm) on certain liver enzymes of 23-week-old rainbow trout.

Days fed	Protein (mg/ml)	G6PDH		NADP-ICDH		NAD-ICDH		LDH		MDH	
		Total[1] activity	Specific[2] activity	Total activity	Specific activity	Total activity	Specific activity	Total activity	Specific activity	Total activity	Specific activity
1	48.0	15.7	0.33	16.9	0.36	0.8	0.016	202	4.26	1450	30.5
	(48.0)[3]	(16.9)	(0.35)	(19.3)	(0.40)	(1.5)	(0.030)	(174)	(3.62)	(1450)	(30.2)
2	43.0	13.3	0.31	16.9	0.40	1.0	0.023	159	3.73	965	22.6
3	49.0	16.9	0.35	20.5	0.42	1.2	0.025	178	3.64	870	17.7
4	46.5	14.5	0.31	16.9	0.36	1.5	0.031	164	3.53	1110	24.0
	(44.0)	(18.1)	(0.41)	(16.9)	(0.38)	(2.0)	(0.045)	(154)	(3.50)	(1060)	(24.0)
5	43.0	12.1	0.28	12.1	0.28	2.0	0.047	140	3.28	965	22.6
6	46.0	9.7	0.21	14.5	0.32	1.2	0.026	135	2.95	820	17.9
	(54.0)	(19.3)	(0.36)	(16.9)	(0.31)	(2.4)	(0.045)	(152)	(2.82)	(1160)	(21.4)
8	46.0	13.3	0.29	13.3	0.29	1.5	0.032	101	2.21	675	14.7
10	50.0	12.1	0.24	15.7	0.31	1.5	0.029	118	2.36	770	15.4
	(44.0)	(21.7)	(0.49)	(21.7)	(0.49)	(2.0)	(0.045)	(174)	(3.92)	(1060)	(24.0)
12	46.0	12.1	0.26	9.7	0.21	2.4	0.052	97	2.10	675	14.7
16	38.0	6.0	0.16	7.3	0.19	1.7	0.045	62	1.64	386	10.1
	(46.0)	(16.9)	(0.37)	(16.9)	(0.37)	--	--	(184)	(4.00)	(1250)	(27.3)
26	39.0	14.5	0.38	12.1	0.31	2.4	0.062	84	2.16	386	9.9
	(44.0)	(16.9)	(0.38)	(19.3)	(0.44)	(2.4)	(0.055)	(164)	(3.74)	(1060)	(24.0)

[1]Total activity values are expressed as μmoles substrate converted per minute per ml of extract.

[2]Specific activity values are expressed as μmoles substrate converted per minute per mg of protein.

[3]Values in parentheses are for paired control samples fed on Diet #1 and assayed on the same day as the experimental diet.

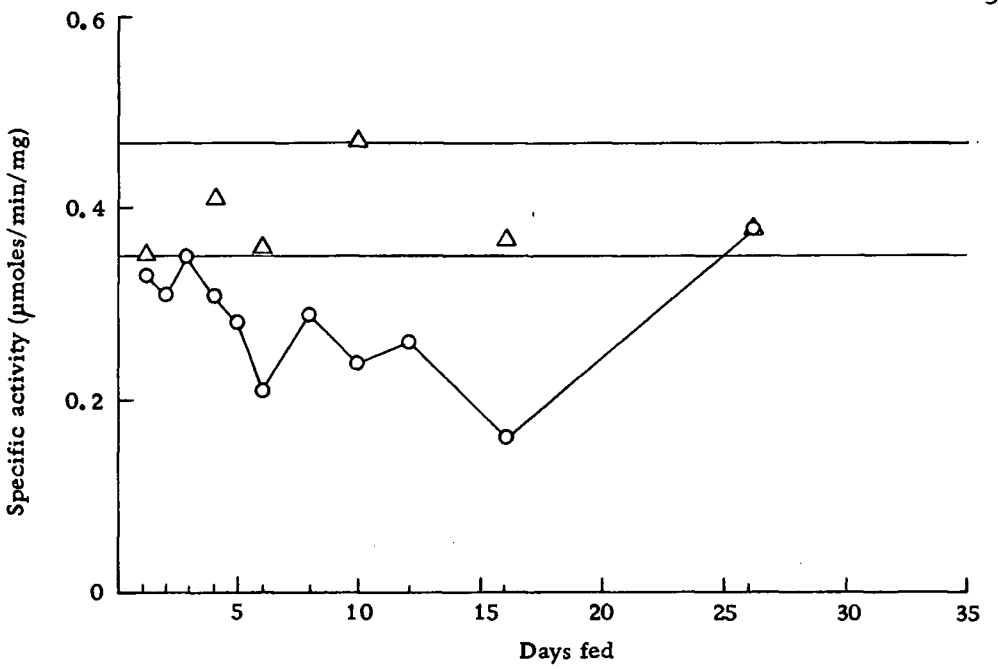


Figure 17. Effect of feeding sterculate at 200 ppm on G6PDH of 23-week-old fish. Triangles are control values. Circles are values for Diet #61-A (200 ppm sterculate). Horizontal lines represent upper and lower limits of the control.

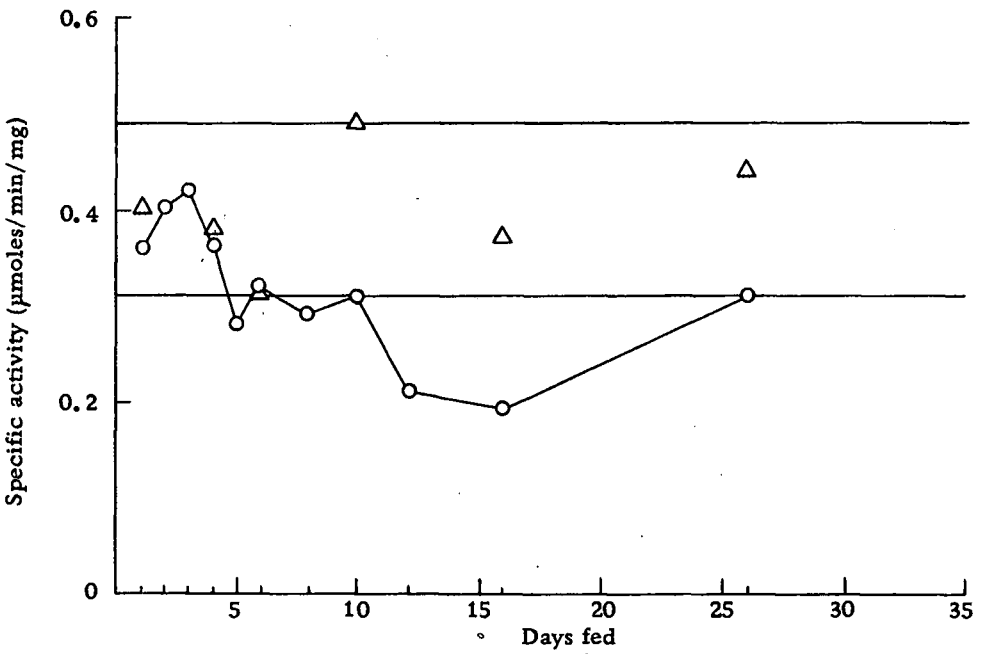


Figure 18. Effect of feeding sterculate at 200 ppm on NADP-ICDH of 23-week-old fish. Triangles are control values. Circles are values for Diet #61-A (200 ppm sterculate). Horizontal lines represent upper and lower limits of the control.

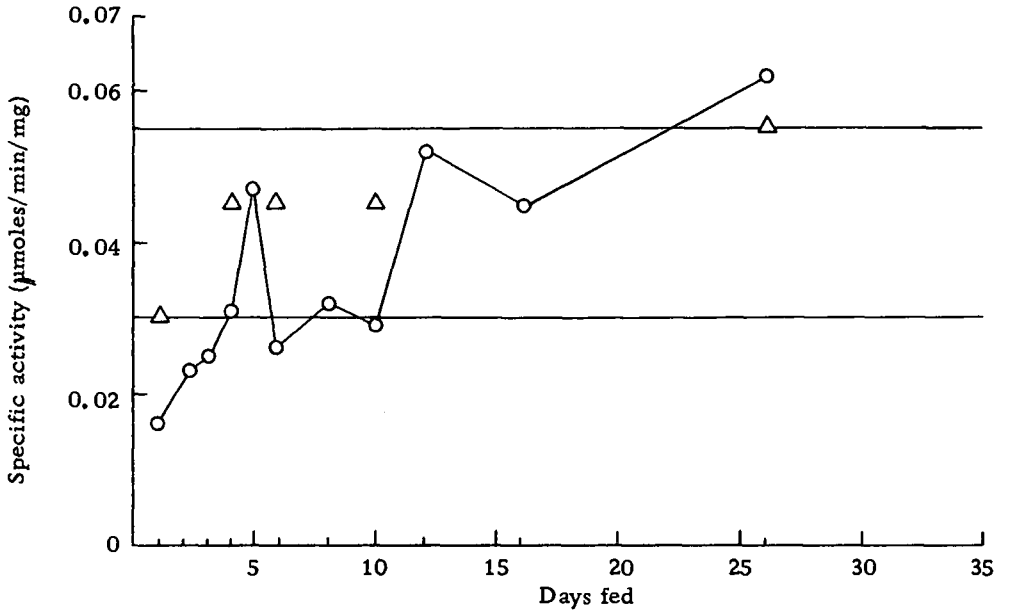


Figure 19. Effect of feeding sterculate at 200 ppm on NAD-ICDH of 23-week-old fish. Triangles are control values. Circles are values for Diet #61-A (200 ppm sterculate). Horizontal lines represent upper and lower limits of the control.

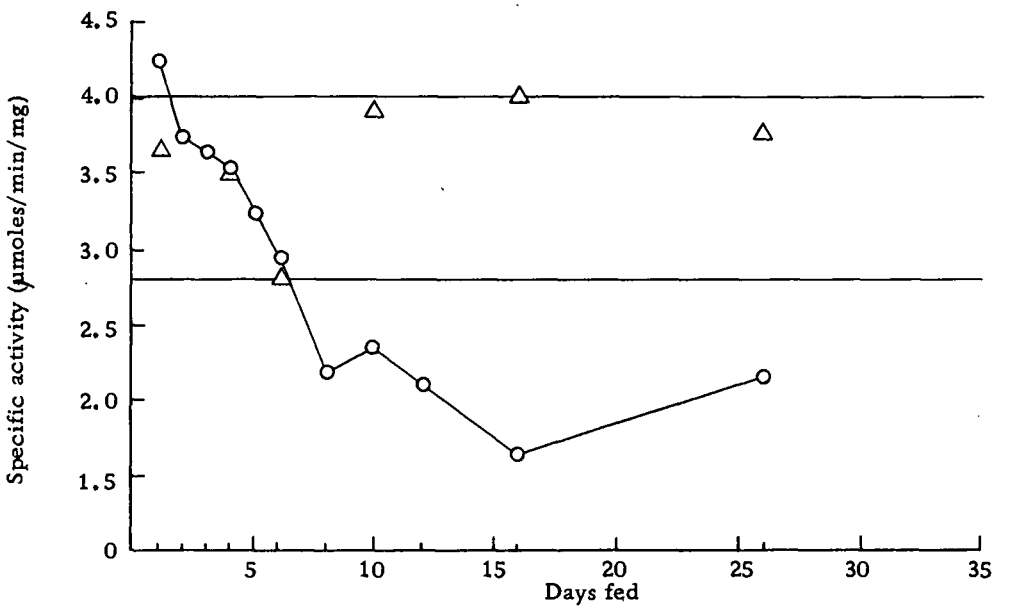


Figure 20. Effect of feeding sterculate at 200 ppm on LDH of 23-week-old fish. Triangles are control values. Circles are values for Diet #61-A (200 ppm sterculate). Horizontal lines represent upper and lower limits of the control.

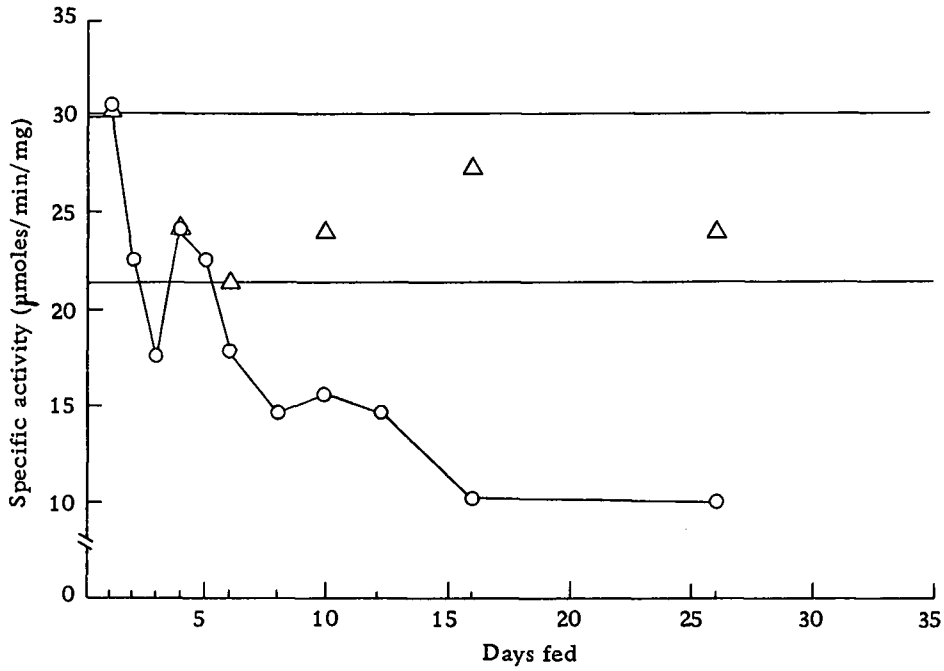


Figure 21. Effect of feeding sterculate at 200 ppm on MDH of 23-week-old fish. Triangles are control values. Circles are values for Diet #61-A (200 ppm sterculate). Horizontal lines represent upper and lower limits of the control.

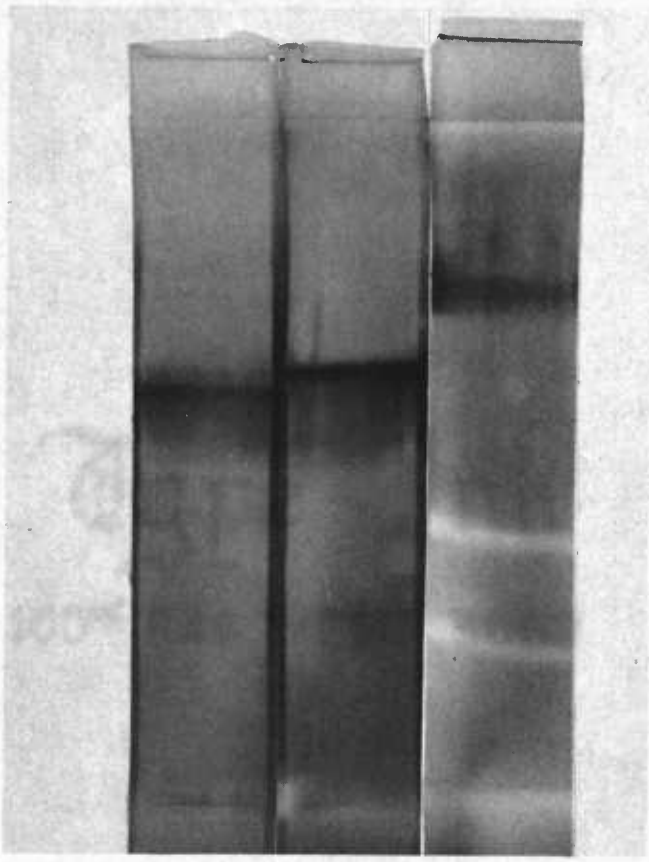
trial from being extended beyond this point. As in Experiment 3, G6PDH specific activity (Figure 17) was lower than the lowest control value from the first day of feeding. Substantial variation from the lower limit was seen from the fifth through 16 days of feeding. After 26 days of feeding, the specific activity of G6PDH was within the control range. With NADP-ICDH (Figure 18), the specific activity remained within the control range until day 12. From 12 to 16 days of feeding, a marked decrease in specific activity below the control values was observed. The specific activity returned to the control range after 26 days of feeding. NAD-ICDH specific activity was initially lower than the control values but was within the normal range after four days of feeding (Figure 19). With LDH, a steady decrease in specific activity was observed throughout the feeding period. The LDH specific activity dropped markedly below the control range after eight days of feeding and did not return to normal before termination of the experiment (Figure 20). For MDH, an initial drop in specific activity was shown after three days of feeding but returned to the control range until the sixth day of feeding. From the sixth day of feeding, a steady lowering of specific activity was noted. No indications appeared that the specific activity of MDH would return to the control level.

Electrophoresis

Throughout Experiments 1 and 2 and at intervals during Experiments 3 and 4, the liver extracts were subjected to vertical

polyacrylamide-gel electrophoresis. The gels were stained and observed for isozymes of G6PDH, LDH, and MDH. Electrophoresis was not successful for ICDH. Wilkinson (126) mentioned that ICDH from most animal tissues is irreversibly inactivated at the high pH necessary for electrophoretic runs. Figure 22 shows the normal isozymic patterns of liver LDH, MDH, and G6PDH of rainbow trout on the control diet. Normally, four bands of MDH, three bands of LDH, and one band of G6PDH were found. The presence of three isozymes of LDH in rainbow trout liver confirms the findings of Massaro and Markert (77) with starch gel electrophoresis. Bouck and Ball (14) found six isozymes of LDH in rainbow trout liver but used a much longer incubation period.

No changes in the isozymic patterns of these liver enzymes were found in Experiment 1 by feeding aflatoxin B_1 at 20 ppb for 35 days. By feeding methyl sterculate at 100 ppm in Experiment 2, a change was noted in the isozymic pattern of G6PDH. After the third day of feeding a second, very faint band of G6PDH, which migrated faster than the principle band found with the controls, was found after a 45-minute incubation period. This band disappeared on the sixth day of feeding and did not appear at any time later in this experiment. In Experiment 3, a four hour incubation period was used for G6PDH and the extra band was found after three days as shown in Figure 23. The additional band showed up intermittently throughout both Experiments 3 and 4. The activity of G6PDH decreased with feeding time so that the amount



A B C

Figure 22. Normal liver isozyme patterns of rainbow trout. Stained for: A - MDH; B - LDH; C - G6PDH.

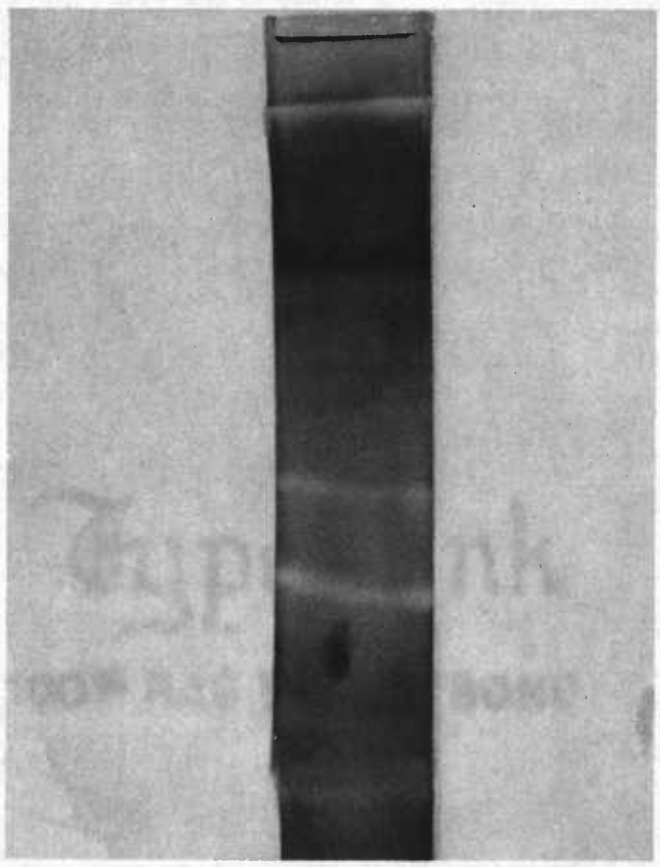


Figure 23. Liver G6PDH isozyme pattern of rainbow trout fed 100 ppm sterculate for three days. Light bands are isozymes of NADPH dehydrogenase.

enzyme placed on the electrophoretic gel decreased, and consequently, the bands of G6PDH became less apparent as the feeding trials proceeded. At times the extra band could not be detected even with a four hour incubation.

Morphological Effects

Livers of rainbow trout fed aflatoxin B_1 (20 ppb) for 35 days in Experiment 1 showed no striking morphological differences from livers of control-diet trout. Figure 24 shows the microscopic appearance of a control-diet liver. After feeding aflatoxin B_1 for 28 days, the liver appeared as shown in Figure 25. Glycogen deposits in both the control and the aflatoxin-fed livers were more numerous than expected from the results of previous studies but this seemed to be characteristic of this particular lot of trout.

The ingestion of methyl sterculate caused distinctive morphological changes in the livers of the rainbow trout. The livers were enlarged, pale, and firm when compared to normal livers. This change occurred quite rapidly becoming apparent after one week of feeding. Microscopic examination of the livers from trout fed sterculate revealed abnormal glycogen deposits within the cell (Figure 26). These deposits became noticeable after six days of feeding at 100 ppm in Experiment 2. Figure 26 shows the appearance of the liver cell after 18 days of feeding 100 ppm sterculate in Experiment 3. The glycogen deposits in this case were quite extensive. Sinnhuber *et al.*

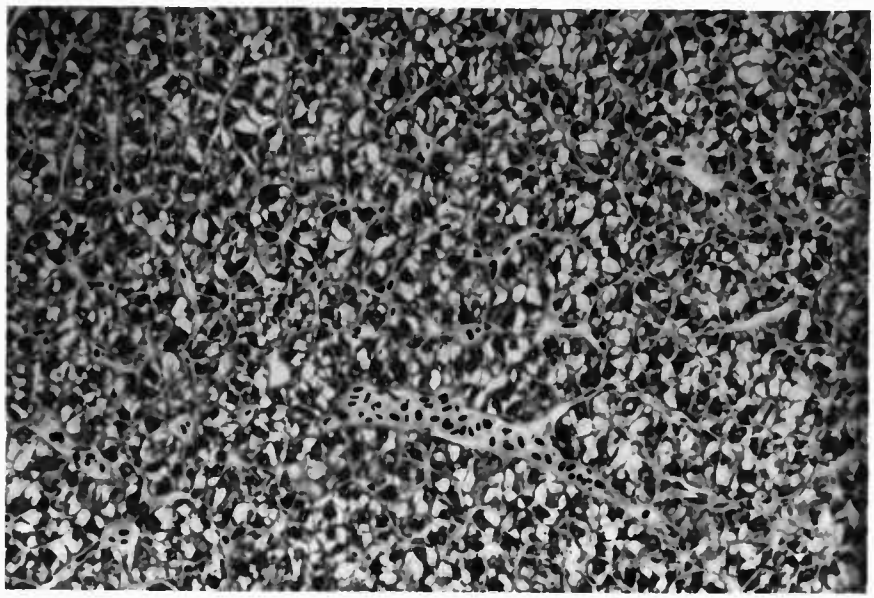


Figure 24. Normal liver cells from trout fed control diet.
Bouin's fixature. H & E, x 128.

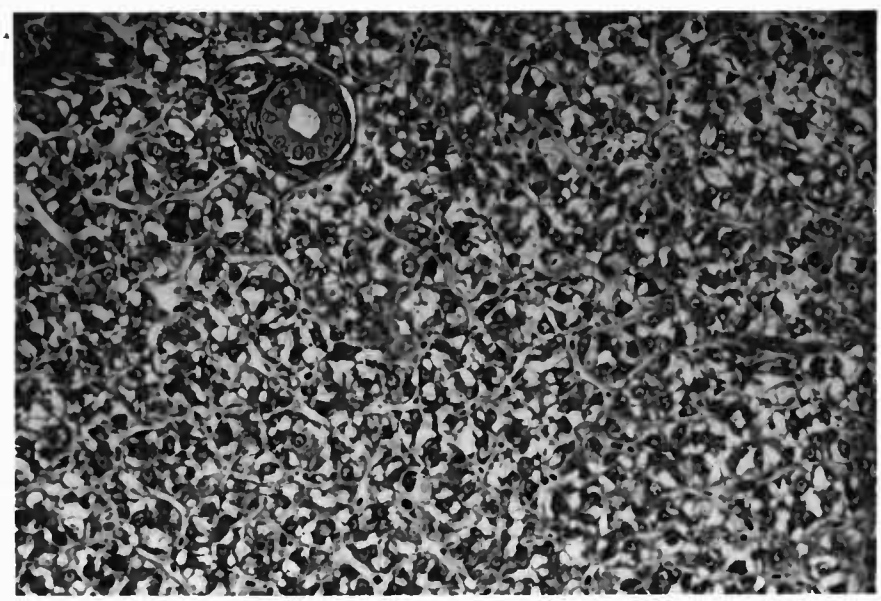


Figure 25. Liver cells from trout fed 20 ppb aflatoxin B_1 for 28 days.
Bouin's fixature. H & E, x 128.

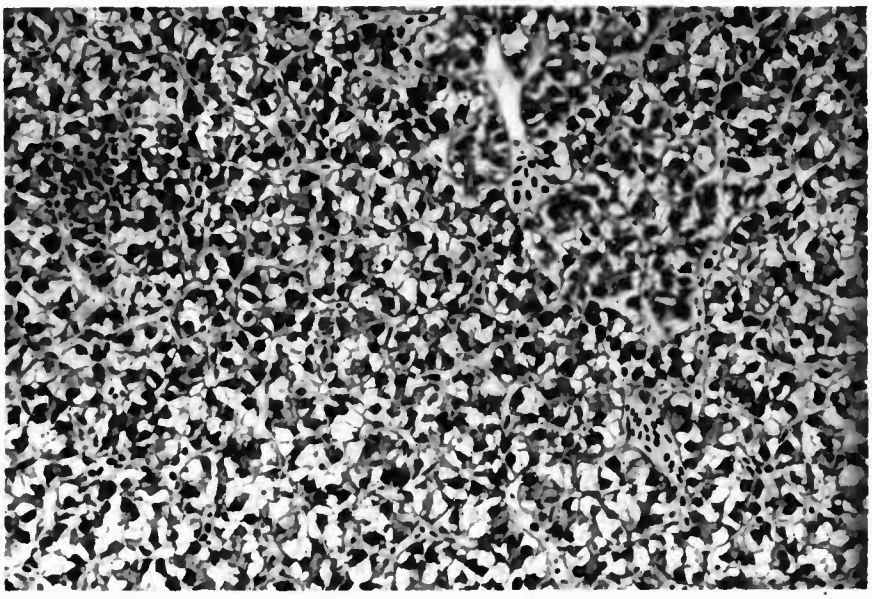


Figure 26. Liver cells from trout fed methyl sterculate (100 ppm) for 18 days (Experiment 3). Bouin's fixature. H & E, x 128.

Figure 27. Liver cells from trout fed methyl sterculate (100 ppm) for 14 days and control diet for three days (Experiment 2). Arrow shows fibrous cell. Bouin's fixature. H & E, x 128.

(112), in a long-term feeding study, noted the appearance of fibrous strands within the cell after feeding CPFA for three months at 400 ppm. Figure 27 shows the liver cell appearance of rainbow trout fed for 14 days at 100 ppm of sterculate and then switched to control diet for three days. Some evidence of these fibers was apparent, although they were not abundant at this early stage of feeding. The fibrous strands were not evident after 14 days on sterculate but became apparent shortly after the switch to the control diet. The glycogen buildup in these livers was also substantial.

DISCUSSION

In Experiment 1, the feeding of aflatoxin B_1 at 20 ppb gave a definite indication of increased specific activity for G6PDH and decreased specific activity for NAD-ICDH. These effects did not appear to be correlated with any changes in isozyme patterns or morphological characteristics of the cell. The specific activities of other enzymes studied, LDH, MDH, and NADP-ICDH, were not affected. Specific activity will be used for comparative purposes here because it reflects the change in enzyme level with regard to protein level whereas total activity would neglect changes in protein concentration.

Aflatoxin B_1 has been shown to cause a high incidence of hepatomas in rainbow trout within one year when fed at a level of 20 ppb (112). It has also been found that feeding aflatoxin B_1 at 20 ppb for 30 days, followed by feeding control diet, results in a similarly high incidence of tumors in one year (66). This finding infers that some deviation from biochemical normality must occur within the cell during the first 30 days of feeding.

Portmann et al. (147) found that both mice and rats metabolized aflatoxin B_1 through a hydroxylation reaction requiring NADPH. Portmann et al. (148) postulated that rats are more susceptible to the carcinogenic action of aflatoxin B_1 than mice because they hydroxylated B_1 more slowly than mice, and transferred B_1 across the cell membranes faster than mice. These authors suggested that B_1 is more

carcinogenic than its hydroxylated derivatives, and that the hydroxylation reaction constitutes a detoxification mechanism.

G6PDH is the rate-controlling enzyme which catalyzes the first reaction in the pentose phosphate pathway. This reaction is also one of the principle reactions in the cell for production of NADPH. It could be postulated, therefore, that G6PDH specific activity is increased because of a requirement for NADPH to carry out the hydroxylation reaction. G6PDH activity would also increase if there was a greater requirement in the cell for the pentose phosphates which are utilized in the formation of nucleic acids. Another possibility would be a repression of some key enzyme in glycolysis which would lead to a greater dependence on the pentose phosphate shunt for glucose metabolism. Further work is needed to determine which, if any, of these possibilities is the cause of the increased specific activity of G6PDH.

As far as NAD-ICDH is concerned, speculation about its decreased specific activity is unwarranted at this point. The enzyme appears to be bound to some other cellular constituent since 75% of the total activity was precipitated by centrifugation at 100,000 x g. Since this study was concerned with only that fraction of the enzyme which was solubilized during homogenization, it is questionable whether the total activity was actually decreased. Therefore, this decrease in total activity could have been due to an effect on degree of binding of the enzyme.

The feeding of methyl sterculate resulted in a decrease in the specific activity of the enzymes of carbohydrate metabolism which were studied. Certain of the enzymes seemed to be more sensitive to the effects of sterculate than others. The specific activity of G6PDH was lowered sooner and to a greater extent than any of the other enzymes. NADP-ICDH and NAD-ICDH were not affected tremendously by sterculate although there was some indication that a slightly lower specific activity existed in the CPFA-fed trout when compared to the control trout. LDH and MDH were intermediate, showing a definite decrease in specific activity but at a later stage than the G6PDH.

The depression of enzyme activity on such a broad scale could have been due to either repression or inhibition of the enzymes. If less substrate were present for these enzymes due to blocking of glycolysis or to a decrease in the amount of glucose available, then repression would be a possibility. However, this does not appear the most likely of the two possibilities. Glycogen deposits were abundant in the livers of sterculate-fed trout, so the initial substrate for glycolysis was present. Also, repression would not normally be exerted on enzymes in three different biochemical schemes unless more than one substrate was lacking. Yet all five of the enzymes studied showed lowered activities.

Evidence has been reported that indicates CPFA will inhibit other enzyme systems. Raju and Raiser (91) and Johnson et al. (56)

showed that CPFA inhibited fatty acyl desaturase in vitro. Raju and Reiser (91) also demonstrated the inhibition of the sulfhydryl enzyme, alcohol dehydrogenase, in vitro and used this as evidence for their contention that CPFA is irreversibly bound to sulfydryls in the active site. Kircher (60) demonstrated an in vitro reaction between methyl sterculate and mercaptans. Johnson et al. (56) claimed however that the inhibition of fatty acyl desaturase may be competitive rather than a noncompetitive reaction with sulfhydryl groups. Since methyl sterculate has been shown to react with sulfhydryls, the decrease in enzyme activity found in this study was likely due to noncompetitive inhibition. The possibility of repression does exist however and a detailed in vitro study is needed to draw a definite conclusion.

Both repression and noncompetitive inhibition could be used to explain the different effects found for the various enzymes. G6PDH, for example, was inhibited at the earliest stage of feeding. This would have been found if G6PDH had available sulfhydryl groups which made it more sensitive to noncompetitive inhibition or if G6PDH was more subject to the repressive effects of substrate and product concentrations than the other enzymes.

Several observations support noncompetitive inhibition as the reason for lowered activity. Firstly, an additional isozyme of G6PDH was found by electrophoresis during the period in which this enzyme was initially inhibited. This additional band could have been G6PDH

with sterculate attached to some site on the enzyme molecule other than the active site. The enzyme would still be active but its electrophoretic mobility would have changed. Secondly, the enzyme activities were found to return to normal after being lowered for only a short time. In Experiment 2 the specific activities of all the enzymes returned to normal after six days of feeding. In Experiment 4 the specific activity of G6PDH and NADP-ICDH returned to normal and the results indicate that LDH and MDH levels would attain normality again. In both Experiment 3 and 4, the specific activities were first lowered then regained their normal levels and finally were inhibited to a greater degree. Noncompetitive inhibition could have been overcome quite easily, since the cell might have synthesized more of the enzyme to counter that which had been irreversibly inhibited. With repression, the level of substrates for the reaction must have increased or product concentration decreased before activity returned to normal. The latter is a possibility but would not likely to have taken place as quickly as enzyme synthesis, particularly since sterculate was fed throughout this period.

The time required for inhibition of the enzymes increased as the age and weight of the liver increased (Experiment 4). The critical factor was probably the level of sterculate in the liver tissue. It appeared that a certain concentration was required to effect noticeable inhibition. Also, the degree of inhibition was similar once inhibition

was effected. Roehm (98) showed that the level of CPFA increases in the liver and other body tissues as feeding continues. In addition, the time required for the dehydrogenase activity to return to normal levels increased with the age of the fish. The reason for this is not known.

Histological observations do not seem to correlate with the inhibition of the enzymes. Microscopic examination of the liver cells after six days of feeding sterculate at 100 ppm in Experiment 1 showed a slight increase in the amount of glycogen. However, enzyme inhibition was observed after only three days. Glycogen continued to build up throughout feeding while enzyme activity returned to normal although Roehm (98) noticed that the large cellular deposits began to disappear after several months. In the present work, fibers form in the cell after several weeks of feeding CPFA (Figure 27). The appearance of these fibers did not appear to be linked to any change in enzyme activity.

Switching the trout to the control diet after feeding sterculate at a level of 100 ppm caused erratic changes in enzyme levels. The protein concentration in the livers dropped markedly (Table 8) and substantial glycogen deposits were formed in the cell rather quickly (Figure 27). The situation appeared to stabilize somewhat after one week but the reasons underlying the glycogen buildup and erratic response are not clear.

The cocarcinogenic activity of sterculate has not been explained.

The reaction of CPFA with sulfhydryl groups and the subsequent inhibition of enzymes may be important in explaining this cocarcinogenicity. Harington (48) hypothesizes that carcinogens may react with -SH groups involved in cell control and division causing inhibition and a subsequent compensatory response leading to overproduction of synthesizing enzymes and eventual loss of control. Methyl sterculate has now been shown to decrease the activity of enzymes involved in carbohydrate metabolism in vivo and it can be speculated that a large number of different enzymes may be inhibited. Although sterculate itself is not carcinogenic, its inhibitory activity in the cell in some respect increases the effectiveness of aflatoxin B_1. It may be that sterculate inhibits the proposed detoxifying hydroxylation reaction for aflatoxin B_1, and, hence, increases the effective concentration of the carcinogen. A long-term feeding study is needed to determine the lasting effects of sterculic acid on these enzymes of carbohydrate metabolism. Also, a study is needed to determine the effects on these enzymes of feeding CPFA and aflatoxin B_1 in the same diet.

SUMMARY AND CONCLUSIONS

The initial effect of both methyl sterculate and aflatoxin B_1 in the diets of rainbow trout on certain dehydrogenase enzymes of carbohydrate metabolism was studied by spectrophotometric assay and vertical polyacrylamide gel electrophoresis. Histological examinations of the liver cells were also performed. The conclusions from these feeding trials are summarized below:

1. The ingestion of aflatoxin B_1 at 20 ppb caused an increase in the level of liver glucose-6-phosphate dehydrogenase (G6PDH) and a decrease in the level of soluble, liver NAD-linked isocitrate dehydrogenase (NAD-ICDH) after 28 days of feeding.
2. A method for electrophoretic separation of the isozymes of rainbow trout liver homogenate was developed. Lactate dehydrogenase (LDH) was found to have three isozymes, malate dehydrogenase (MDH) normally had four isozymes, and one isozyme of G6PDH was found.
3. Feeding sterculate at either 100 ppm or 200 ppm caused a decrease in the specific activity of G6PDH, LDH, MDH, NADP-ICDH, and NAD-ICDH. This decrease in specific activity was postulated to have been due to a noncompetitive inhibition, although repression was also considered to be a possibility.
4. The decrease in specific activity was, in certain cases, followed

by a recovery to normal levels after several days. The time required for this recovery depended upon the particular enzyme, the age of the fish, the weight of the liver, and the level of sterculate fed.

5. G6PDH appeared to be more sensitive to the effects of sterculate than the other enzymes studied. ICDH was comparatively resistant to the inhibitory action of methyl sterculate while LDH and MDH were intermediate.

6. The length of feeding time necessary to cause a decrease in specific activity also depended upon the age of the fish, the weight of the liver, and the level fed. It was postulated that a critical level of sterculate must be deposited in the liver tissue before noticeable inhibition of the enzymes could take place.

7. Methyl sterculate in the diet also caused certain morphological changes in rainbow trout livers. Glycogen deposits were noted after several days of feeding and, in one trial, fibrous strands of unknown origin began appearing in the cells after several weeks. The livers were enlarged, firm, and pale in comparison to normal livers.

8. An additional band of G6PDH was found by electrophoresis after feeding sterculate for a short time. The significance of this additional band is not known yet.

9. The cocarcinogenicity of CPFA with aflatoxin B_1 is likely due to

the inhibitory action of CPFA in the cell although the exact mechanism remains unknown.

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APPENDIX

APPENDIX A

SPECTROPHOTOMETRIC ASSAY METHOD

The substrate solutions were made and stored in the refrigerator for a maximum of four days. Bulk lots of these reaction mixtures were usually made to increase the reproducibility of the weighing procedures. The bulk lots were made as follows:

LDH Reaction Mixture

0.0088 g sodium pyruvate (1×10^{-2} M)

0.0170 g DPNH (1.6×10^{-3} M)

24 ml distilled water

26 ml 0.05 M Tris-HCl buffer (pH 7.5)

MDH Reaction Mixture

0.0106 g DPNH (8×10^{-4} M)

0.0053 g oxalacetic acid (5×10^{-3} M) - by dissolving in 7.0 ml of water and adding 1.0 ml of 0.1 N NaOH

20 ml distilled water

22 ml 0.05 M Tris-HCl buffer (pH 7.5)

NADP-ICDH Reaction Mixture

0.0093 g trisodium isocitrate (6×10^{-3} M)

0.0228 g TPN (5×10^{-3} M)

0.1352 g $MnSO_4$ (0.1 M)

20 ml distilled water

30 ml 0.05 M Tris-HCl buffer (pH 7.5)

NAD-ICDH Reaction Mixture

0.0046 g trisodium isocitrate (6×10^{-3} M)

0.0798 g DPN (2×10^{-2} M) - by dissolving in 4.8 ml of H_2O and 1.2 ml of 0.1 N NaOH

0.0676 g $MnSO_4$ (0.1 M)

7 ml distilled water

12 ml 0.05 M Tris-HCl buffer (pH 7.5)

G6PDH Reaction Mixtures

0.0219 g disodium glucose-6-phosphate (1.2×10^{-3} M)

0.0152 g TPN (5×10^{-3} M)

0.1624 g $MgCl_2$ (0.1 M)

18 ml distilled water

32 ml 0.05 M Tris-HCl buffer (pH 7.5)

These solutions were kept cool at all times to increase their storage time. During spectrophotometric runs the solutions were immersed in an ice bath which kept them near refrigeration temperature. The solutions were allowed to warm to room temperature immediately before making a run.

The enzymes extracts were prepared as noted in the text. Trout livers were removed, perfused, and immediately frozen in liquid nitrogen. After transport to the laboratory, the frozen livers were weighed and a 1:5 homogenate was made in 0.01 M Tris-citrate buffer. This buffer was prepared by dissolving 0.121 g Tris in 50 ml of water, adding 3 ml of 0.1 M citric acid (2.10 g citric acid · H_2O in 100 ml H_2O), and adjusting the pH to 7.0 with 0.1 M citric acid. Homogenization was performed in a micro container attachment (Eberbach Corp.) to a Waring Blendor for one minute at a rheostat setting of 90. The homogenate was centrifuged at 2500 x g for 10 minutes in a Sorvall Superspeed centrifuge in a cold room (4° C). Four ml of this supernatant was centrifuged at 100,000 x g for 60 minutes in a Beckman Model

L-2 refrigerated ultracentrifuge. This supernatant was then diluted 1:50 with distilled water to yield the enzyme extract used for the spectrophotometric assays. A 1:100 dilution was prepared to use in assaying total protein content and MDH.

The total enzyme activity was determined by measuring the change in absorbance at 340 mμ in a Beckman DB recording spectrophotometer. The first step was always to zero the spectrophotometer and the recorder by using two quartz cuvettes filled with water. The recorder was set at a speed of one inch per minute, and, since absorbance was being measured, the log scale was utilized. Each of the cuvettes was then filled with 2.5 ml of the reaction mixture for the enzyme to be assayed. The spectrophotometer was then zeroed. In the case of LDH and MDH, where reduced cofactor was used, the slit width was manually opened to 2 mm. For the others, a narrow slit setting was sufficient. To the reference cuvette, 0.5 ml of distilled water was added. The reaction was initiated by adding 0.5 ml of enzyme solution to the sample cuvette. In the case of MDH, 0.5 ml of distilled water was added to the sample cuvette and the reaction was started by adding 0.05 ml of the 1:100 enzyme extract. With the G6PDH, NADP-ICDH, and NAD-ICDH assays, which utilized oxidized cofactors, the sample cuvette was placed in the sample slot of the spectrophotometer. For LDH and MDH assays, which required reduced cofactors, the sample cuvette was placed on the reference side of the spectrophotometer. The addition of enzyme was considered as the zero time for the reaction. The reaction time was measured with a stopwatch. The sample cuvette was inverted five times to thoroughly mix the enzyme with the substrate. This procedure was essential to obtain a straight line in many cases. The reaction was linear for several minutes but the change in absorbance between 30 and 45 secs. was used to calculate the activity of the enzyme. The activity was calculated according to the following formula:

$$\frac{\frac{\Delta A \times 3}{6.22 \times 10^6} \times \text{dilution factor}}{\text{mg protein/ml}} = \mu\text{moles substrate converted/min/mg}$$

Protein was measured by the E_{280}/E_{260} method (22) using 3.0 ml of the 1:100 dilution of the enzyme extract in the sample cuvette and distilled water in the reference cuvette.