EFFECT OF AFLATOXIN B_1 ON MITOCHONDRIAL FUNCTION

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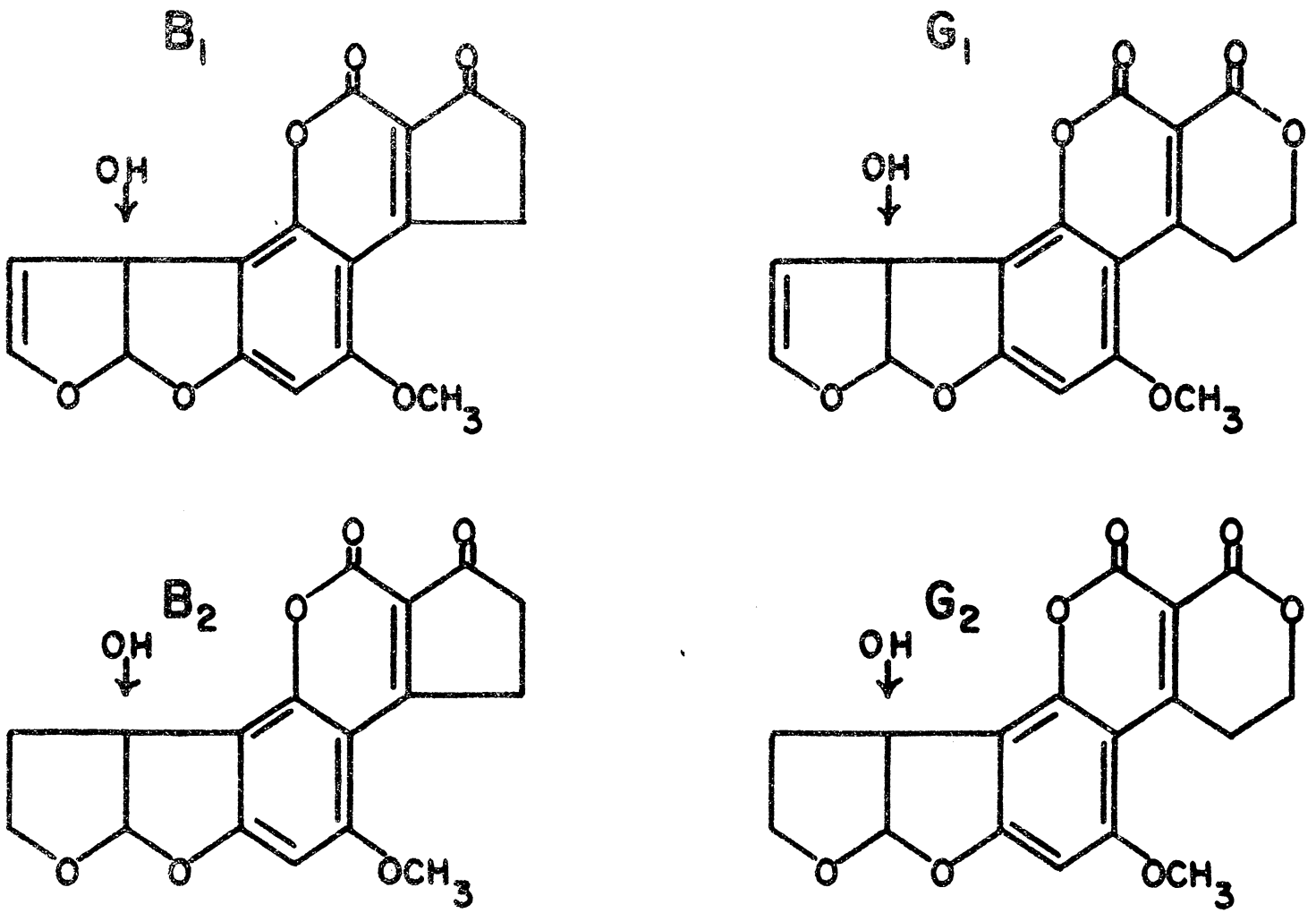
ABBREVIATIONS

AFB_1	- Aflatoxin B_1
ADP	- Adenosine Diphosphate
ATP	- Adenosine Triphosphate
DNA	- Deoxyribonucleic Acid
RNA	- Ribonucleic Acid
DNP	- 2, 4-Dinitrophenol
X°	- X degrees Centigrade
NAD	- Nicotinamide Adenine Dinucleotide
NADH	- Reduced NAD
DMF	- Dimethylformamide
EDTA	- Ethylenediaminetetra-Acetic Acid
Hepes	- (N-2-Hydroxyethylpiperazine-N-2-ethane sulfonic acid)
TMPD	- N, N, N', N'-Tetramethyl-p-phenylene-diamine Dihydrochloride
ATPase	- Adenosine Triphosphatase (EC 3.6.1.4.)
FAD	- Flavin Adenine Dinucleotide
CMC	- Critical Micelle Concentration
mRNA	- Messenger Ribonucleic Acid
P_i	- Inorganic phosphate

Structure of Aflatoxin Showing Sites of Hydroxylation.



AFLATOXIN

INTRODUCTION

Aflatoxin, a complex of highly substituted coumarins, is elaborated by various fungi of the genus *Aspergillus*. It is frequently found as a contaminant in foods and feeds. The peanut and products manufactured from it, especially peanut butter, are the most frequently contaminated foods [1]. The mycotoxin has also been isolated from wheat, rice, soybean, corn, bread, milk, and cheese [2]. *Aspergillus flavus* is the principal organism responsible for the production of aflatoxin. It has been shown that the *Aspergilli* are not important as parasites in growing grain [3]. They are, however, abundant in the air of grain elevators, especially large terminal elevators. Aflatoxin therefore is important as a hepatocarcinogen in areas of the world that are damp and have poor storage facilities [4].

The problem of mycotoxins in the food chain did not come into sharp focus until 1960, when contaminated peanut meal was fed to turkeys in England and the deaths of large numbers ensued. This phenomenon was reported in the literature as the turkey X disease [5]. Since this incident aflatoxin has been shown to be an extremely potent, species specific hepatocarcinogen. This is in addition to its extreme toxicity [6-9].

Many different derivatives of aflatoxin are elaborated by various fungi. Aflatoxin B_1, G_1 and their dihydrofuran derivatives, B_2 and G_2, are the most common. These compounds can be separated chromatographically and have characteristic R_F values. B_1 and B_2 fluoresce

blue under long range ultraviolet light while G_1 and G_2 fluoresce yellow-green [10]. There are also a number of hydroxylated aflatoxins produced by fungi. The point of hydroxylation is carbon number four [11]. The oral seven day LD_{50} in one day old ducklings, based on a 50 g body weight is: B_1, 18.2 µg; B_2, 84.8 µg; G_1, 39.2 µg; and G_2, 172.5 µg [12]. This has been verified by other workers [3]. The LD_{50} dose of aflatoxin B_1, the most toxic of the aflatoxins, varies with age, sex, and strain of the animal. For male rats weighing 100 g it is 7.2 mg/kg of body weight [14].

The carcinogenicity of aflatoxin also varies with the age, sex, and species of the animal. The most carcinogenic aflatoxin is aflatoxin B_1 and it can produce hepatomas in rainbow trout at a minimum concentration of one part per billion [15, 16]. The study of the biological effects of aflatoxin can therefore be justified because of its economic importance and its extreme carcinogenicity. Two million dollars were lost by the peanut industry in Virginia last year because of aflatoxin infestation. The carcinogenic properties of aflatoxin B_1 also affords an excellent opportunity to study the early biochemical events in carcinogenesis.

Biochemical Effects of Aflatoxin B_1

The shift in the ultraviolet spectrum has been the primary criterion employed to demonstrate the binding of AFB_1 to DNA. It has been reported that there is a shift in the absorption maximum from 363 nm to 366 - 368 nm produced by the binding of AFB_1 to calf-thymus

DNA [17]. This shift is accompanied by marked hypochromicity at 362 nm. On the basis of the spectral shift described above, it was calculated that 600 moles of native DNA-phosphorus bound one mole of AFB_1. The binding of the aflatoxins B_1, G_1, and G_2 to calf-thymus DNA has also been studied [18]. Quantitatively similar spectral shifts were produced when these compounds bind to DNA. The shifts however varied in magnitude. The largest is seen with B_1, intermediate values are obtained with G_1, and the smallest values are obtained with G_2. The extent of toxicity and carcinogenicity of these compounds is proportional to the magnitude of the spectral shifts induced. Further studies of the spectral shifts associated with the interaction of aflatoxins with nucleosides showed that the purine bases and the amino group are responsible for the binding of aflatoxins to DNA [19]. These workers also showed that the DNA-AFB_1 complex could by separated by chromatography on Sephadex G-50 indicating noncovalent binding. Black and Jirgenson used equilibrium dialysis to determine the extent of binding of aflatoxin B_1 to calf-thymus histones and DNA [20]. Their results indicated that aflatoxin binds to DNA and also to lysine rich histones under the conditions employed. This binding was associated with an increase in the viscosity of the histone and DNA solutions. It was concluded that the binding of aflatoxin results in conformational changes in these molecules. Histones are important in the control of mRNA synthesis and in DNA synthesis as well [21, 22]. This change in histone conformation could result in the synthesis of mRNA not normally expressed at a given time.

Net DNA synthesis is also inhibited by AFB_1 when it is administered to hepatectomized rats [23]. It has also been shown that administration of 100 µg of aflatoxin inhibits the incorporation of thymidine into liver DNA by as much as 95%. These workers also showed that AFB_1 had no direct effect on the enzymes involved. The mode of action appeared to be the binding to the DNA and subsequent inhibition of primer activity [24]. Wragg and co-workers have shown a decrease in the DNA content of *E. coli* grown in the presence of AFB_1. This was shown to be due to the inhibition of DNA polymerase activity. Primer activity of DNA grown in the presence of the toxin was also reduced [25].

Aflatoxin B_1 has also been shown to inhibit RNA polymerase. Portman and Campbell were the first to show that inhibition of RNA polymerase could occur *in vitro*. They were able to obtain 78% inhibition with rat liver chromatin instead of the DNA preparations used by other workers. They used rat liver DNA as well and found no inhibition. All experiments were implemented with *E. coli* RNA polymerase. Their work brought into sharp focus the importance of histones in the interaction of AFB_1 with the genetic material [26].

Wogan showed that chromatin isolated from rats injected with 0.5 mg/kg of AFB_1 reduced the activity of RNA polymerase by 28 to 46% when the enzymes were isolated from untreated animals. *Escherichia coli* RNA polymerase was able to function on the template isolated from treated animals unimpaired. If RNA polymerase is prepared from animals that have been treated with AFB_1 and assayed using calf-thymus

DNA and rat liver chromatin as a template, the enzymes from treated and untreated animals had the same activity on both templates. It would therefore seem that the toxin has no direct effect on the enzyme. Addition of AFB_1 at levels of 5 - 12 µg/ml of medium to an *in vitro* assay system containing intact rat liver nuclei, isolated from untreated rats, does not inhibit RNA polymerase activity. Controls indicate that the lack of inhibition was probably due to the impermeability of the nuclear membrane to AFB_1. These results seem to indicate that the inhibitory effects of AFB_1 on rat liver nuclear RNA polymerase activity *in vivo* results from the interaction of the toxin with some component of the chromatin and not from direct action on the enzyme [27].

Mitochondrial Structure

The outer and inner mitochondrial membranes complement each other perfectly in function. The outer membrane is freely permeable to a wide range of charged and uncharged substances with molecular weights up to 10,000. This includes all acids oxidized by the mitochondria and, among larger substances, inulin. The primary function of the outer mitochondrial membrane is to support enzymes such as rotenone-insensitive NADH: cytochrome c reductase and to serve as a boundary for the loosely bound enzymes found in the interspace. An important implication of this concept is that all low-molecular weight, charged components present in isolated mitochondria (including nuclectides) are located inside the inner membrane [28,29].

The inner mitochondrial membrane is, however, another matter. Extensive studies based on measurements of the space occupied by various substances present in the medium in relation to the total water space of the mitochondria, and correlated with morphological observations, have led to the conclusion that the inner mitochondrial membrane possesses only very limited permeability to most substances. Those excepted are uncharged molecules of a molecular weight not greater than 100 - 150. The charged molecules of physiological importance pass through the inner membrane by way of specific exchange mechanisms [30,31,32]. If the mitochondrion were capable of taking up substrates on a nonexchange basis, the osmotic pressure would destroy it. The exchange mechanisms for the uptake of substrates are extremely important to the viability of the mitochondrion. If one wishes to characterize a mitochondrial inhibitor that is hydrophobic and has a molecular weight greater than 150, it then becomes important to consider the location of its site of action. If it is inside the inner membrane, the permeability of the membrane to the inhibitor must be considered.

The studies of Palade were the first to indicate that there were morphological differences between the outer and inner membranes. He called the folds in the inner membrane cristae [33]. It remained for Klingenberg to observe that the cytochrome content of various mitochondria was proportional to the number of cristae [34]. This observation placed the cytochromes and the electron transport system in the inner membrane. Klingenberg and Pfaff then grouped mitochondrial enzymes on

the basis of their solubility. Those dissociable only by surface active agents were considered to be structurally bound. This includes cytochromes b and c_1, cytochrome oxidase, succinate dehydrogenase, NADH dehydrogenases, and to some extent ATPases. Those dissociable by sonic oscillation were considered to be tightly enclosed. These included NAD, all the NAD-dependent dehydrogenases of the Krebs cycle and the transaminases [35,36,37]. These enzymes are found in the matrix [38,39]. The enzymes that are easily dissociable ionically under hypotonic conditions are cytochrome c, phosphate transferase, and ADP-ATP exchange enzymes [29,39]. These enzymes are considered to be loosely bound to the outside of the inner membrane.

The identification of knobs on the matrix side of the inner mitochondrial membrane gave rise to the theory of non-identity of the two sides of the inner membrane [40,41]. Many workers feel that the best means of determining the sidedness of the inner membrane is through the use of submitochondrial particles. Lee and Ernster state that the insensitivity of submitochondrial particles produced by sonication to the reversal of oxidative phosphorylation in high magnesium concentrations is due to the fact that the magnesium is on a different side of the inner mitochondrial membrane [42]. The sonication process is schematically represented in Figure 10. In intact mitochondria, high magnesium concentration inhibits reversal of oxidative phosphorylation [43]. This explanation could also be used to explain reverse respiratory control found in Gregg particles [44]. In these submitochondrial particles made by sonication of rat liver mitochondria, inorganic

phosphate stimulates respiration while ADP depresses it. This is again the opposite of that found in intact mitochondria. More direct evidence obtained by electron-microscopy indicates that these particles form vesicles that have the head pieces or knobs pointing out [45,46]. This again is opposite to what would be found in intact mitochondria.

It is now of interest to examine the location of the electron transport system in the inner membrane. In the literature, the knobbed side of the membrane or matrix side is referred to as the M side. The outside or the side that contains cytochrome c is referred to as the C side. The schematic in Figure 21 may help to clarify this rather complex topology. NADH dehydrogenase and succinate dehydrogenase are on the M side of the inner membrane [47,48]. Cytochrome b is also found on the M side of the inner membrane [48,49]. Cytochrome a_3 is found on the M side of the membrane [50]. There is some controversy concerning the location of cytochrome oxidase. This is caused by the fact that reduced cytochrome c is oxidized on both sides of the inner membrane [51]. This has been explained by placing cytochrome a near the C side of the inner membrane and cytochrome a_3 near the M side and giving both of them the ability to oxidize reduced cytochrome c. Cytochrome a_3 is definitely on the M side of the inner membrane but more work is needed to exactly determine the location of cytochrome a. Cytochrome c_1 is said to be found on the C side of the membrane [49]. Since immunological techniques were used to determine the location of cytochrome c_1, there is a possibility of confusion with cytochrome c. The location of cytochrome c seems the most certain. It is located on the C side of the inner membrane [52,53,54].

Mitochondrial Inhibitors

Many compounds are known to inhibit mitochondrial oxygen consumption; though, not all of them have been thoroughly characterized. Among the most thoroughly characterized inhibitors is antimycin. Antimycin is elaborated by the Streptomycetes family of microorganisms, and it binds quite firmly to mitochondria and mitochondrial fragments. It has a sigmoidal inhibition curve and about 0.07 μmoles per mg of protein gives maximal inhibition [55], though less is routinely used. The site of action of antimycin is known to be the second crossover point, i.e., between cytochrome b and cytochrome c_1 [56,57,58]. High concentrations of antimycin lowers the P:O ratio of rat liver mitochondria oxidizing succinate by 30% [59]. Antimycin at a concentration of 0.1 mM also partially inhibited the NADH: Ubiquinone oxidoreductase, succinate: Ubiquinone oxidoreductase, and cytochrome c: oxygen oxidoreductase systems [60]. Rotenone, a plant product, also inhibits mitochondrial oxygen consumption. A level of 0.033 nmoles/mg of rat liver mitochondrial protein inhibits the oxidation of L-glutamate by 65% [61]. Rotenone, like antimycin, binds very tightly to its specific site in mitochondria. Rotenone at a level of 30 nmoles/mg of mitochondrial protein has been shown to completely inhibit the oxidation of NAD-linked substrates by rat liver mitochondria. Rotenone at a level of 0.1 nM has no effect on the oxidation of succinate. Excess rotenone also has no effect on the P:O ratio and DNP does not remove the inhibition caused by rotenone [62,63,64].

Amytal or amobarbital is a derivative of barbituric acid. Among

its properties is the ability to combine with flavin and adenine [65, 66]. At a concentration of 1.8 nM amytal completely inhibits the oxidation of NAD-linked substrates [67]. A partial inhibition of the oxidation of extramitochondrial NADH was also observed whereas no inhibition of succinate was observed. Ernster *et al.* also found that rotenone inhibited the rat liver mitochondrial P_i-ATP exchange reaction only slightly while the DNP-stimulated ATPase activity and the P:O ratio resulting from the oxidation of succinate remains unaffected [68]. These functions were inhibited by amytal, even though the concentration required for these effects were generally higher than those required for the inhibition of NAD-dependent dehydrogenase [62]. Rotenone and antimycin differ from amytal in that amytal can be very easily removed from mitochondria and mitochondrial fragments while rotenone and antimycin are removed only with great difficulty [69]. Chance and Ernster feel that both rotenone and amytal inhibits at the same site, i.e., between a flavoprotein (FPD_1) on the NAD side of the site, reduced by NADH but not by succinate, and flavoproteins (FPD_2 and FP_s) on the cytochrome b side of the site that can be reduced by succinate in the presence of ATP [70].

Effects of Coumarin Type Compounds on Mitochondrial Function

Coumarin-type compounds have long been known to show marked effects on mitochondria. The stimulation of respiration (uncoupling) by dicoumarol on rat liver mitochondria has been extensively studied [71].

This derivative is also known to cause respiratory inhibition as shown by direct inhibition of succinate dehydrogenase and 3-hydroxybutyrate dehydrogenase [72]. Moreover, it has been reported that ochratoxin A, a derivative of dihydroisocoumarin and a fungal metabolite produced primarily by *Aspergillus ochraceus*, markedly inhibits coupled respiration of rat liver mitochondria [73].

Aflatoxin, a coumarin-type compound elaborated by *Aspergillus flavus*, has been reported to affect mitochondrial function, although the reports are inconsistent. Clifford and Rees [74] reported that liver mitochondria from rats administered 7 mg (AFB_1)/kg body weight remained unaffected for up to 24 hours in their respiratory capacity and P/O ratios when a range of substrates were used. These results contrast to those of Svoboda *et al.* [75,76], who showed that both phosphorylation and oxygen consumption were decreased in mitochondrial preparations of rats dosed with 0.45 mg AFB_1/kg body weight prior to sacrifice. Moreover, these latter results are similar to those of Brown and Abrams [77] and Brown [78] who examined the toxic effects of AFB_1 on the liver mitochondrial function of chickens and ducklings.

The disagreement above concerning the effects of AFB_1 on mitochondrial function is probably due to species differences in the permeability of the inner mitochondrial membrane. The difference in the susceptibility of different strains of rats to mitochondrial impairment by AFB_1 is also probably due to differences in permeability of the inner mitochondrial membrane.

Hepatoma Metabolism

The phenomenon of aerobic glycolysis in cancerous tissues was first observed by Warburg in 1923 [79]. Warburg proposed that cancer originated as a result of injury to the respiratory system which resulted in an increase in glycolysis with lactic acid production and a decrease in oxygen consumption. In 1956, Warburg [80] elaborated on his hypothesis by comparing the aerobic and anaerobic production of lactic acid both by ascites cancer cells and by nonneoplastic embryonic tissue. He showed that with chorionic cells the anaerobic production of lactic acid was rather high and was suppressed completely in the presence of oxygen. In contrast, the ascites tumor cells showed an even higher anaerobic glycolysis and, although lower, it was retained in the presence of oxygen. Furthermore, the consumption of oxygen was lower in the ascites cells as compared to the chorion preparation. He maintained that this role of glycolysis is a necessary process for the survival of cancer cells and the primary events of cancer start when the normal cells adapt to the anaerobic metabolism after impairment of their normal respiratory processes [81]. Further, he claimed that this impairment of respiration is irreversible and that the cause of this injury is lack of energy. This theory has been widely questioned largely because of the absence of a direct correlation with genome aberration. It was not however considered that the aberration could be in the mitochondrial genome. Since every glycolytic enzyme found in tumor tissue is coded for on the nuclear genetic material, a perturbation in control caused by the inactivation of

mitochondria through mutation could result in the expression of glycolytic isozymes which are not normally found in the liver [82,83, 84,85].

The glycolytic isozyme pattern varies as a function of the degree of differentiation of the hepatoma [85]. Well differentiated, slow growing tumors differ moderately from normal liver tissue in their glycolytic isozyme pattern, while poorly differentiated, fast growing hepatomas have a vast change in their glycolytic isozymes [83]. The growth rate of both Novikoff and Morris hepatomas, the two most extensively studied hepatomas, increases as the degree of differentiation decreases [86]. Normal liver isozymes are almost completely absent in poorly differentiated hepatomas, and, as with most other tumors, they then resemble each other more than they resemble the tissue of origin [87].

Poorly differentiated, fast growing hepatomas have a 5 to 10 fold increase in hexokinase with its very low Km for glucose as opposed to glucokinase with its very high Km for glucose. Glucokinase is predominate in normal livers. Normal liver is highly oxidative and uses fatty acids as its fuel. However, in poorly differentiated, fast growing hepatomas, glucose is the primary fuel. This marks a return to enzyme patterns characteristic of the fetal liver [88]. Tumors with this type of pattern have a very high rate of glycolysis [89], and are not sensitive to the diet, insulin, or other hormonal controls. This seems reasonable because glucokinase is the enzyme which would normally respond to hormonal control in the liver [90].

Aldolase, which exists in multiple forms, has four subunits with different primary structures [91,92]. Two predominant forms are found: A, or muscle aldolase, and B, or liver aldolase. It is interesting to note that fast growing, undifferentiated hepatomas have the anaerobic or muscle aldolase [93]. Pyruvate kinase, another important enzyme in the glycolytic pathway, also exists in multiple forms. Two major forms are found in the liver: The A form, again much like the enzyme found in muscle, and the B form, which is predominant in the normal liver [94,95]. The latter is responsive to a carbohydrate diet and insulin treatment, and thus is commensurate with normal liver function. Needless to say, poorly differentiated hepatomas are not capable of carrying on gluconeogenesis. In the fast growing Novikoff and Morris hepatomas, the muscle type enzyme replaces the normal liver enzyme, and it is found in quantities 4 to 5 times higher than in the normal liver. This would stimulate glycolysis and decrease the cellular ADP concentration.

Pyruvate kinase seems to be one of the more likely candidates for control or loss of control in poorly differentiated tumors. Phosphofructokinase has been thought to be one of the rate moderating steps, but the addition of fructose diphosphate to whole respiring homogenates of the well differentiated tumors resulted in a low yield of ATP and lactate [96]. Respiratory ATP was not affected. When fructose diphosphate was added to the poorly differentiated homogenate, glycolytic ATP production increased and the typically rather poor respiratory ATP production stopped completely.

The oxidation of fatty acids also varies with the degree of differentiation of the tumor. The oxidation of palmitate, butyrate, and β-hydroxybutyrate by homogenates of well differentiated tumors is somewhat lower than the normal liver. However, the oxidation by poorly differentiated tumors was negligible [97]. It would also appear from work with Morris hepatomas that the glycerol phosphate shuttle is progressively lost as the tissue becomes more poorly differentiated [98]. The reason for this reduced function of mitochondria is not entirely understood; although it has been shown that the level of fatty acid activating enzymes is high in well differentiated tumor mitochondria while they are low or absent in poorly differentiated tumors [99].

There are certain phenomena which appear to be common to most tumor mitochondria. The NAD effect is one of them [100]. This effect is characterized by low respiratory capacity of tumor homogenates or tumor mitochondria. This however is not a true effect of tumor mitochondria, but it is an effect seen in energy depleted mitochondria in general. There are three possibilities for the maintenance of NAD within mitochondria: (a) bound to highly specific sites, (b) bound to non-specific sites, or (c) merely retained by a selectively permeable mitochondrial membrane. It is believed that all three of these phenomena may occur [101]. Lehninger has shown that mitochondria cannot oxidize externally added NADH unless the mitochondria were first treated with distilled water [102]. Hunter and Ford [103] have shown that P_i, Ca^{++} and deoxycholate along with many other agents lead to an inactivation of NAD dependent oxidations. Simultaneously there

was a loss of material having an absorption maximum at 260 nm from the mitochondria. NAD, when added to the test system, replaces 60 to 90% of the phosphorylation ability. The capacity to oxidize NAD-linked substrates can be restored to mitochondria depleted of NAD by the addition of ATP and magnesium chloride. These mitochondria contain only 10 to 20% of their initial NAD but are capable of oxidizing pyruvate and malate with a P:0 ratio of 2.44 and with a respiratory control of from 3 to 6 [104]. Such a high respiratory control ratio would lead one to believe that considerable mitochondrial integrity has been preserved. It would appear that the permeability of NAD to mitochondrial membranes is inextricably involved with the respiratory capacity of mitochondria or their ATP content [105].

Mitochondria from tumors are much more heterogeneous than mitochondria from normal tissues [106,107,108]. Tumor mitochondria also tend to be much smaller in size. Overling and Bernard have described the mitochondria of tumor cells as quite variable in shape, size, and density, and they have further stated that there are usually less mitochondria per cell than is seen in normal tissue [109,110]. This could be explained on the basis of mitochondrial mutation. It has recently been found that melanoma mitochondria have reduced amounts of a protein that has been characterized as band 4 on polyacrylamide gel electrophoresis. Since the protein of this band seems to be a major product of mitochondrial protein synthesis [111], it would certainly lead to structural instabilities in these mitochondria. Proteins from the inner membrane of hepatomas were also found to be missing

some of its major protein bands which have been shown to be coded for on the mitochondrial genome [112]. This could account for the great fragility of tumor mitochondria, and possibly for their reduced activity. This would also implicate the genetic mechanism of the mitochondria in the process of carcinogenesis.

Comments on the Literature Review

As can be seen in the previous literature review, aflatoxin affects a great many cellular processes. It is as yet uncertain which of these is the primary carcinogenic event, if indeed it is reasonable to think in terms of a primary carcinogenic event. The process of carcinogenesis may well be a number of sublethal events all of which co-operatively result in neoplasia. The possibility that the primary event involves the induction of a prophage must also be considered. This could either occur in the nucleus or in the mitochondria. The induction of the prophage would not necessarily involve the direct action of aflatoxin on the genome. It could result from the perturbation of cellular metabolism altering the level of some effector.

The researcher at this point in time may choose the operating premise that he feels is the most valid. At present there is strong feeling that viruses are the universal cause of cancer. This may or may not be true of some cancers, but it has not been shown for all of them. It certainly has not been demonstrated that hepatomas are caused by viruses. If indeed they are viral in nature, the conditions for induction of a prophage would be the primary carcinogenic event, and

a valid field of inquiry.

One of the striking problems indicated in the preceding review is the permeability of the various organelle membranes to aflatoxin. The nucleus is especially interesting. Intact nuclei under *in vitro* conditions are not permeable to aflatoxin. Yet the evidence is overwhelming that they are *in vivo*. This clearly implicates a cytoplasmic component, probably a protein, in the transport of aflatoxin across the nuclear membrane. This is reminiscent of the transport of steroids [113,114,115]. The structural similarities between aflatoxin and steroids is obvious. The transport of aflatoxin across nuclear and mitochondrial membranes should in the future be a very profitable field of inquiry.

OBJECTIVES

The phenomenon of aerobic glycolysis in cancerous tissue has been observed by many investigators since its original description by Warburg in 1923. Its significance, however, has been widely questioned largely due to the absence of any obvious relationship with genetic aberration. The extensive changes in glycolytic isozyme patterns resulting in a strong glycolysis have been shown to be in agreement with this phenomenon, but the primal lesion causing the alterations in cellular metabolism remains unexplained.

The hepatocarcinogen aflatoxin B_1 has been shown by many workers to effect mitochondrial function. If it could be demonstrated that mitochondrial function is adequately disturbed to result in a drop in cellular ATP levels, the control process within the cell would undoubtedly be disturbed. Once more the repair and synthesis of mitochondrial DNA might also be affected. One might reasonably expect a high incidence of mitochondrial mutations under these conditions. Inactivation of mitochondria by mutation might be the sought after primal lesion.

The purpose of this study was to examine the affect of aflatoxin B_1 on the electron transport system, and to identify, if possible, its site of action. It was also considered important to know the effect of aflatoxin B_1 on phosphorylation efficiency. Studies were also undertaken to examine the effect of some dietary variables on these parameters.

MATERIALS AND METHODS

Materials

Rats: Male Sprague-Dawley derived rats weighing 90-150 g were used in all experiments unless otherwise indicated. All rats were purchased from Flow Research Labs, Dublin, Virginia.

Feed: All rats were fed Wayne Lab Blox purchased from Allied Mills unless otherwise indicated.

Chemicals: AFB_1 was purchased from Calbiochem, LaJolla, California. Purity was checked by measurement of its ultraviolet spectrum in methanol or chloroform, and its chromatographic homogeneity in 2 solvent systems (chloroform: acetone, 90:10; upper phase of benzene: ethanol: water, 46:35:19) Adsorbosil-5 was used as the thin layer chromatographic adsorbent and was purchased from Applied Science Laboratories, State College, Pennsylvania. Hepes buffer, DNP, ADP (disodium salt), and rotenone were purchased from Sigma Chemical Company, St. Louis, Missouri. They were stored under desiccant until used. TMPD was purchased from Eastman Kodak Company, Rochester, New York. It was recrystallized routinely from ethanol prior to use. DNP was recrystallized from diethyl ether. Menadione was purchased from Merck and Company, Inc., Rahway, New Jersey. Ascorbate, DL-methionine, vitamin diet fortification mixture, vitamin-free casein, and thiamine free diet were purchased from Nutritional Biochemical Corp., Cleveland, Ohio. Mazola corn oil was purchased locally from Radford Brothers, Blacksburg, Virginia. Sucrose was obtained locally from Virginia Polytechnic Institute and State

University Food Stores for feeding experiments. All other chemicals were reagent grade.

Methods

Preparation of Mitochondria

Mitochondria were isolated from 90-150 g male Sprague-Dawley derived rats by a slightly modified method of Johnson and Lardy [116]. The liver was minced with scissors and homogenized as 4 g of tissue brought to 50 ml of 0.05 M Hepes buffer (pH 7.4) containing 0.25 M sucrose and 0.002 M EDTA. Instead of 1 centrifugation at 600 x g, the crude homogenate was first filtered through glass wool and then centrifuged twice at 100 x g for 10 min with the supernatant being filtered each time through glass wool. The mitochondria were then pelleted by centrifugation at 14,500 x g for 10 min. The buff-colored mitochondrial pellet was then carefully resuspended so as not to include the bottom-layered erythrocytes and grayish nuclear material. The mitochondria were then washed once in the homogenization solution and the final resuspension was made in the oxygen electrode solution described below. This fractionation routinely yielded 10-12 mg of mitochondrial protein per g of liver. Respiratory control ratios between 4.8 and 6.0 were consistently obtained when succinate was used as a substrate. The mitochondria were used immediately after isolation and the respiratory control ratio was obtained before and after each experiment to insure mitochondria integrity [117].

Measurement of Oxygen Consumption

Oxygen consumption was measured potentiometrically in a Beckman Model 777 oxygen analyzer with a Clark electrode. The electrode was housed in an all-glass 3-ml reaction vessel which was enclosed in a water jacket; water was circulated at 30°. The oxygen concentrations were traced on a Honeywell Electronik 194 recorder with 0-100 mv input. Calibration of the oxygen analyzer and recorder were accomplished by methods described by the manufacturer,

The oxygen electrode solution was principally that of Estabrook [117]. The final volume was 3.0 ml and it contained 0.25 M sucrose; 0.01 M potassium phosphate, pH 7.4; 0.005 M $MgCl_2$; 0.02 M KCl; 0.025 M Hepes buffer, pH 7.4; and 0.002 M EDTA. Mitochondria were suspended in the solution and were added in a volume of 0.1 ml containing approximately 1 mg protein.

Protein was estimated after each experiment by the method of Lowry *et al.* [118].

Determination of AFB_1 Critical Micelle Concentration

The method for determining the (CMC) of AFB_1 was essentially that of Anderson and Anderson [119] and it was determined with a dual beam Unicam SP-800A UV/visible spectrophotometer. The wavelength employed to measure light scatter was 420 nm. Cuvettes with a 3-ml volume and 1-cm light path were employed and the cell compartment was maintained at 30°. The AFB_1 was added in dimethylformamide to the oxygen electrode solution in the cuvette. The amount of dimethylformamide added with the

AFB_1 was the same as that amount added when the AFB_1 was employed as a mitochondrial inhibitor.

By-pass of the Second Coupling Site

This system is based on the principle that TMPD has the ability to oxidize cytochrome b and reduce cytochrome c or c_1 [120]. In the process the second coupling site is bridged. TMPD also has some other properties that have to be considered. Among them is the fact that TMPD is capable of oxidizing NADH non-enzymatically. The effect however is minimal since glutamate can be oxidized in the presence of TMPD with a P:O of two. Still it is best to use succinate as well as an NAD-dependent substrate to be confident of the data. Ernster [121] has recommended the use of 0.3 mM TMPD for the by-pass of the second coupling site. In these experiments either 0.1 mM or 0.2 mM TMPD is adequate to effect the by-pass. The lower concentration also minimized interactions with NAD.

Assay of the Third Site of Phosphorylation

The procedure of Sanadi and Jacobson [122] was employed to route electrons through the third site of phosphorylation. Ascorbate (1.5mM) was used to reduce TMPD (1 mM) which, in turn, can reduce cytochromes c and c_1. Cytochrome oxidase then oxidizes cytochrome c. Rotenone was used to suppress oxidation of endogenous fatty acid, but in these experiments it made no difference. There appears to be a minimal contribution made by endogenous substrate in these experiments.

Determination of the ADP:O Ratio

The ADP:O ratio was determined by the polarographic method of Estabrook [117]. The mitochondria are placed in the oxygen electrode solution previously described in the absence of ADP. The oxygen consumption during state 4 is monitored until a rate is determined. A known amount of ADP is then added, and the state 3 rate is monitored until the ADP is exhausted. The state 4 rate is again assumed by the mitochondria and it is monitored until the rate is established. The oxygen consumed during the period of state 3 metabolism is then computed from the recorder tracing. The amount of oxygen consumed is then divided into the ADP added and the ADP:O ratio results.

Preparation of Gregg Particles

The mitochondria employed in the preparation of Gregg particles were isolated by the method previously described. Forty mg of mitochondrial protein were suspended, after their second washing, in 25 ml of Hepes buffer (pH 7.4). The mitochondria were then subjected to one 15 min sonic treatment using an amount of energy equal to that described by Gregg in his second procedure [44]. The instrument used was a model W185 Sonifier Cell Disrupter, with the micro tip, manufactured by Heat Systems-Ultrasonics, Inc., Plainview, New York. The plate voltage was set at 5. Energy was determined by the rise in temperature of a known volume of water over a given period of time. A mixture of acetone: ethanol (1:1) was employed as a coolant-300 ml total volume in a 400 ml beaker wrapped with urethane foam. Solid carbon dioxide about the size

of a pea, was added to the coolant mixture to maintain the temperature external to the sample tube at -6°C. This kept the temperature of the sample at 0°-5°C. The sample was placed in a 50 ml Sorvall stainless steel centrifuge tube. The coolant mixture was stirred constantly by means of a magnetic stirrer. If care is taken to maintain the coolant temperature at -6°C, the sample temperature will remain at 2°C. After sonication the suspension was centrifuged at 25,000 X G for 20 min in a Sorvall RC2-B centrifuge. The resulting amber colored suspension was then centrifuged at 144,000 X G for 30 min in a Beckman L2-65B ultracentrifuge. The liquid was then decanted, and the particles were resuspended in 0.025 M Hepes (pH 7.4) and 0.25 M sucrose. The particles were then frozen in small aliquots; they remained stable for about 3 weeks. About 20% of the original protein was retained in this preparation.

pH Determination

The pH for most experiments was determined using a Sargent model DR pH meter manufactured by E. H. Sargent & Co., Chicago, Illinois.

Oxygen Consumption Using Gregg Particles

The oxygen sensing apparatus employed is the one previously described in this Methods section. The reaction medium contained: 10 mM substrate; 10 mM Hepes buffer (pH 7.4), 3.0 mM Mg Cl_2, 15 mM potassium phosphate (pH 7.4), and 0.1 mg of particle protein. When β-hydroxybutyrate was used as a substrate, 0.2 mM NAD^+ was added. The

temperature was maintained at 30°C. Oxygen consumption was linear until 60% of the oxygen had been consumed. Cytochrome c stimulated oxygen consumption when β-hydroxybutyrate was the substrate, but not when succinate was the substrate. The amount of cytochrome c that should be added depends on the preparation [44].

Dinitrophenol Stimulated ATPase Activity in Gregg Particles

The reaction mixture contained 10 mM ATP (pH 7.4), 10 mM Hepes (pH 7.4), 0.3 mM DNP, 10 mM succinate, 1.0 mM Mg Cl_2, 25 µg of particle protein, 0.2 mM NAD, and 10 mM β-hydroxybutyrate. The materials were incubated at 30°C for 10 min in a total volume of 2 ml. A time course indicated that the protein was stable for this length of time. The reaction was stopped with 0.2 ml of 4.0 M trichloroacetic acid. Inorganic phosphate produced is then determined by a modification of the Fiske-Subbarow Method detailed below [123]. One-half g of amino-2-napthol-4-sulfonic acid is added to a solution of 15% $NaHSO_3$ and made up to 185 ml. Five ml of 20% Na_2SO_3 is then added to the above solution and the suspension is warmed until most of the solids have gone into solution. The suspension is then filtered and stored in an amber glass bottle. This is the Fiske-Subbarow Reagent. Other reagents needed are 10 N H_2SO_4 and 2.5% ammonium molybdate. The assay mixture contained 0.4 ml of 10 N H_2SO_4, 0.8 ml of 2.5% ammonium molybdate and 0.4 ml of Fiske-Subbarow Reagent. Sample and water are added to a total volume of 10 ml. The optical density is determined after 20 min at 660 nm. A reagent blank including protein was employed. Protein was removed by centrifugation before phosphate determination.

Spectrophotometric Determination of the Oxidation Reduction Level of Cytochrome b and cytochrome c

The average oxidation state of cytochromes b and c were determined using a dual beam spectrophotometer. The turbid cell compartment of a Unicam SP-800 spectrophotometer was employed. Gregg particles were used because the excessive turbidity of a mitochondrial suspension could not be handled by the available equipment. The particles were suspended in the solution used in the oxygen uptake experiment. The reference cuvette contained oxidized particles, no substrate, while the sample cuvette contained reduced particles, 10 mM β-hydroxybutyrate, 0.2 mM NAD. Three ml cuvettes with a 1 cm light path were employed. AFB_1 was added to both cuvettes at a time when the oxygen concentration was still high. The optical density was taken after the addition of AFB_1 at 430-410 nM for cytochrome b and 550-540 nM for cytochrome c [124]. The values obtained before the addition of AFB_1 were subtracted from the values obtained after its addition.

Protein Deficiency Experiment

Protein deficiency was accomplished by feeding weanling rats, 55 to 60 g, of the type described previously, a 5% casein diet for 15 days. The composition of the 5% diet is as follows: sucrose, 846 g; vitamin free casein, 50 g; corn oil, 40 g; salt mix, 40 g; vitamin mix, 22 g; DL-methionine, 2 g. The composition of the 20% diet is as follows: sucrose, 696 g; casein, 400 g; corn oil, 80 g; salt mix, 80 g; vitamins, 44 g; DL-methionine, 4 g. Animals on the 5% diet were fed *ad libitum*.

A group fed the 20% diet were pair-fed to the group fed the 5% diet. This was to account for caloric deprivation. A group was also fed the 20% diet *ad libitum*. Tap water was given to all groups *ad libitum*. All animals were sacrificed after 15 days [125].

Thiamine Deficiency Experiment

Thiamine deficiency was accomplished by feeding weanling rats, 55 to 60 g, of the type described previously a thiamine deficient diet prepared by the Nutritional Biochemical Corp. Control animals were fed Wayne Lab Blox. Both groups were sacrificed after 20 days.

RESULTS AND DISCUSSION

The critical micelle concentration of AFB_1 was determined in the oxygen electrode solution to reflect the experimental conditions used for the mitochondrial inhibition studies. These data are shown in Fig. 1. The critical micelle concentration of 0.48 mM applies to these conditions only, since modifications of ionic strength and sucrose concentration could cause alterations in this important parameter.

The hydrophilic nature of AFB_1 suggests the possibility that it might bind to the Teflon membrane, the O-ring or the plastic head of the oxygen electrode. The results shown in Fig. 2 illustrate that very little AFB_1 binds to any portion of the oxygen sensing apparatus. A total of only 22 µg remains unextractable after 5 min when 250 µg of AFB_1 is added at 0 time. This experiment was done in the absence of mitochondria. Moreover, in experiments undertaken to measure state 3 inhibition, AFB_1 was added to an oxygen electrode cell already containing mitochondria so that the onset of inhibition was measured within 1-2 min, thereby further minimizing the significance of any artifacts caused by binding of AFB_1 to the apparatus. It has therefore been concluded that any reduction of the effective concentration due to AFB_1 becoming bound to the sensor apparatus is negligible.

Fig. 3 shows a representative experiment on the effect of AFB_1 on the consumption of oxygen by state 3 mitochondria during the metabolism of glutamate and succinate. As can be seen, both succinate and glutamate are inhibited equally by 0.48 mM AFB_1. This indicates that the point of

Figure 1. Determination of the critical micelle concentration under conditions employed in experiments with whole mitochondria.

Repeated 3 different times.

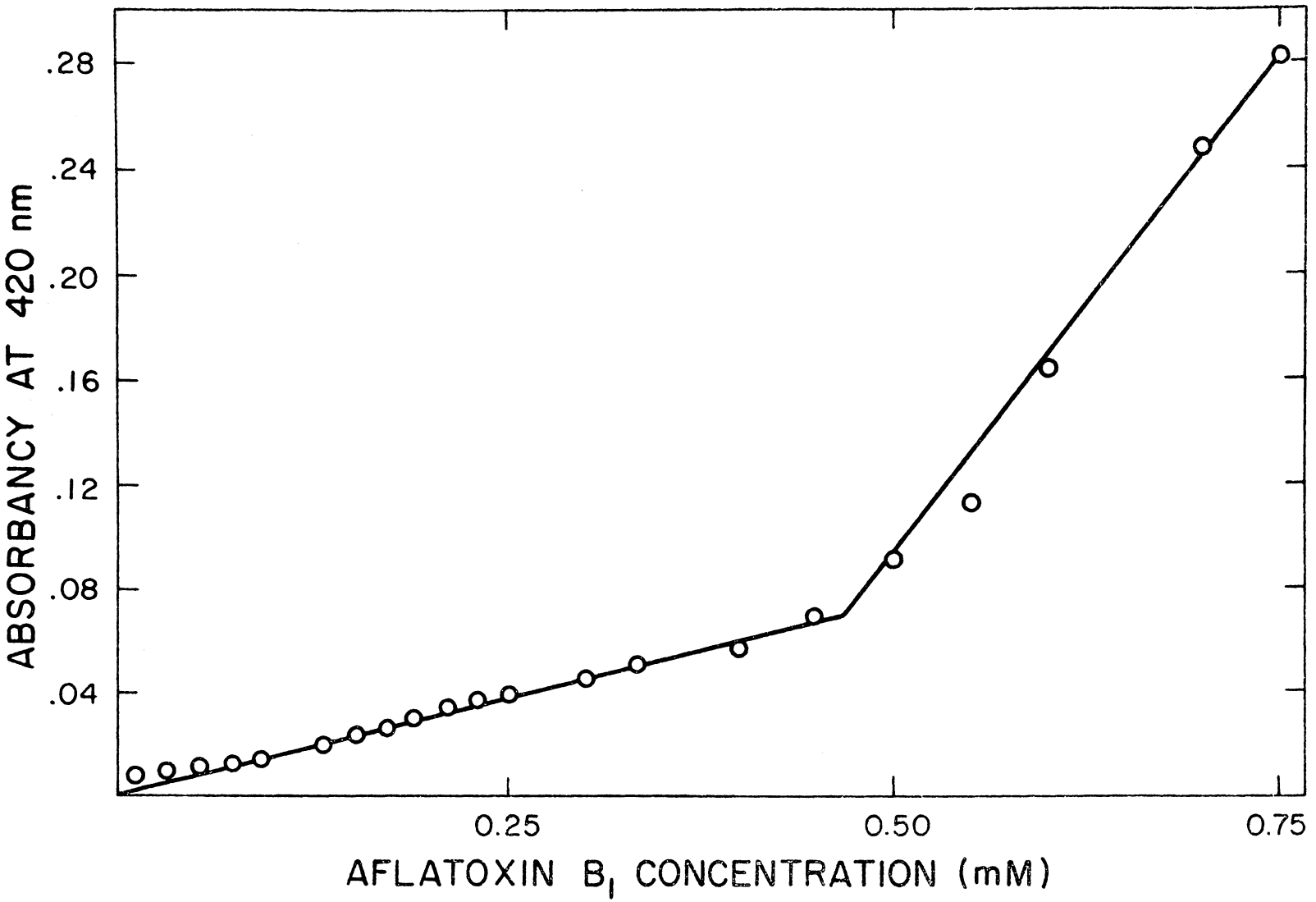


Figure 2. Binding of aflatoxin B_1 to the oxygen electrode.

Results of 3 experiments. The bars indicate standard error.

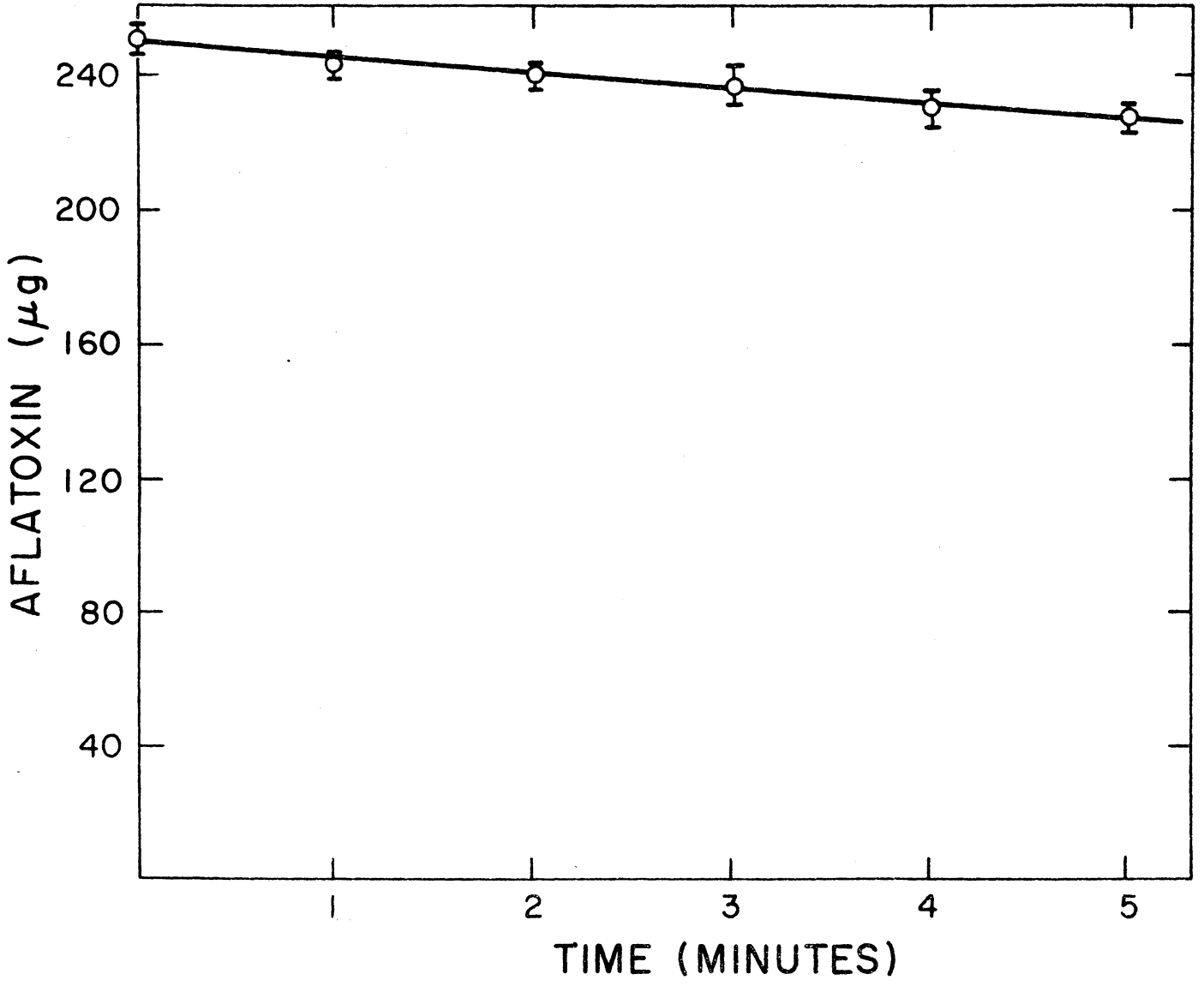
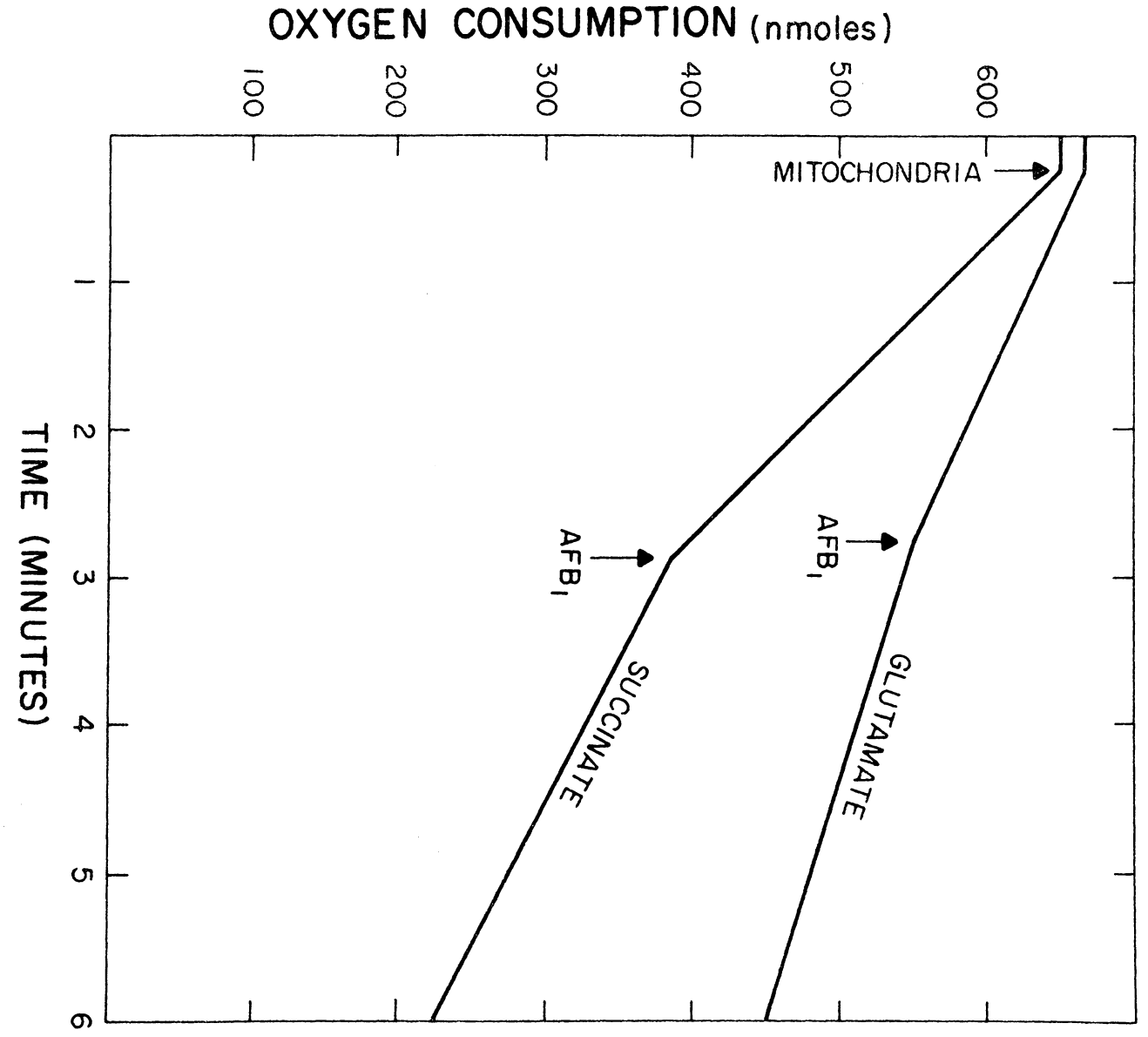


Figure 3. Inhibition of glutamate and succinate oxidation by aflatoxin B_1 in whole mitochondria.

This representative experiment has been repeated over ten times. 5mM succinate and 5 mM glutamate were present.



inhibition is in that portion of the electron transport chain that is common to both substrates, i.e., between cytochrome b and cytochrome oxidase.

Fig. 4 shows the effect of AFB_1 on oxygen consumption expressed as per cent of control. The inhibition rises linearly until the CMC is reached, and then the inhibition levels off. The most striking feature of the curve is that it plateaus within error at the CMC. Interpretation of the inhibition beyond the CMC is not possible since the effect of the micelles may be different from the effect of the individual molecules. It is known, for example, that NAD and FAD bind to micelles [119]. It is also known that micelles denature dehydrogenases [119]. The membranes of mitochondria can themselves be considered as micelles and it would seem reasonable that micelles could affect mitochondrial structure. No specific conclusions will therefore be drawn above the CMC.

The results shown in Fig. 5 indicate that the state 3 inhibition remains unaffected between 3 and 10 weeks of age. It was not anticipated that the age of the animals would be a source of error in these experiments, but since the AFB_1 structure resembles a steroid hormone, maturation of the animal may influence its transport across organelle membranes.

Fig. 6 shows the variation in the inhibition of oxygen consumption with protein concentration at a constant level of AFB_1. The inhibition remains essentially independent of the amount of protein over the range shown. At quantities of mitochondrial protein below 0.5 mg per 3.0 ml,

Figure 4. Inhibition of oxygen consumption as a function of the aflatoxin B_1 concentration using whole mitochondria.

Result of more than 4 experiments on 4 different days. The bars indicate standard error. This curve was obtained with either 5 mM succinate or glutamate.

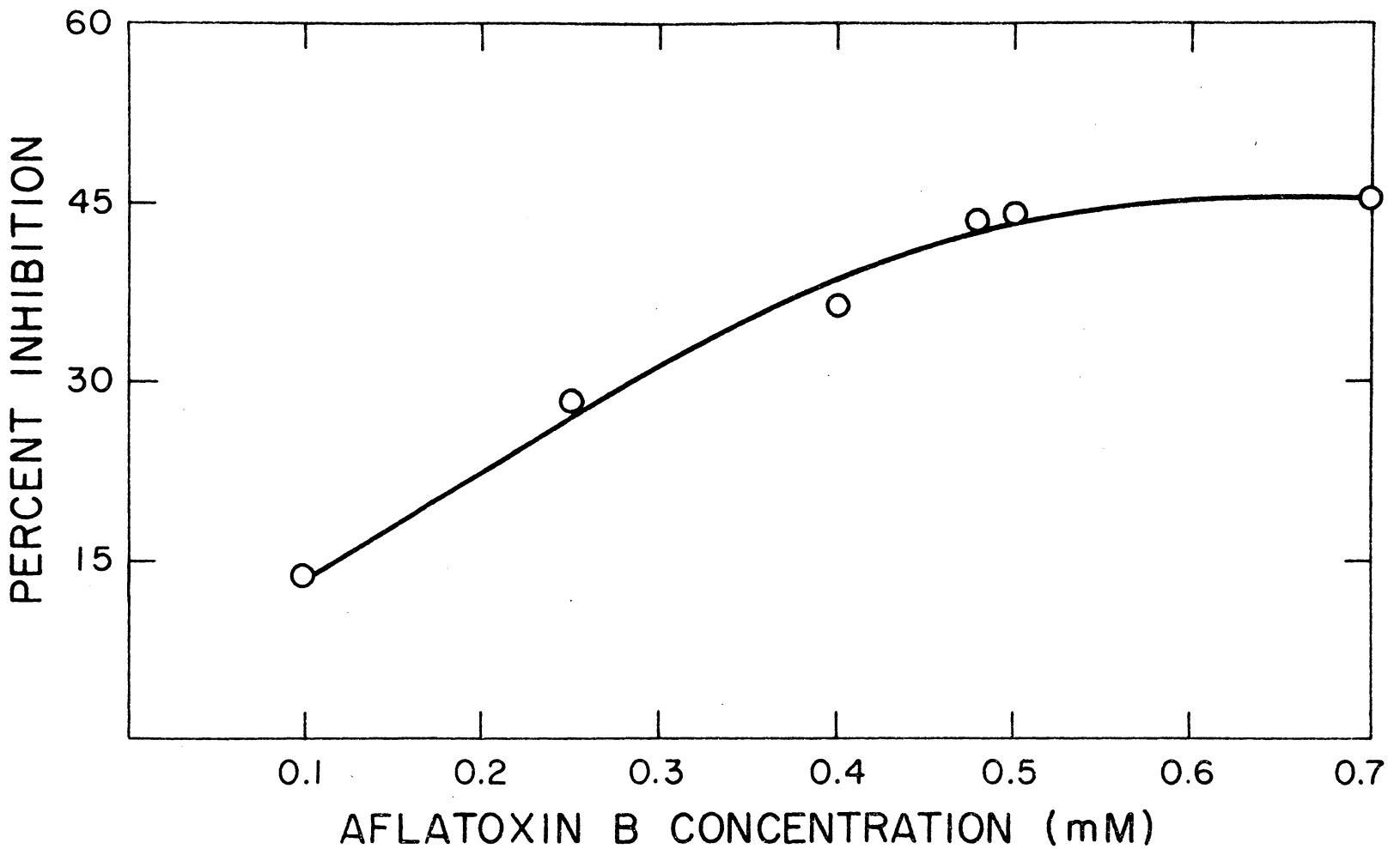


Figure 5. Inhibition of oxygen consumption by aflatoxin B_1 as a function of the age of the animal from which the mitochondria were isolated.

Result of 3 different experiments on 3 different days. The bars indicate standard error. AFB_1 was present at 0.25 mM. 5 mM succinate was employed.

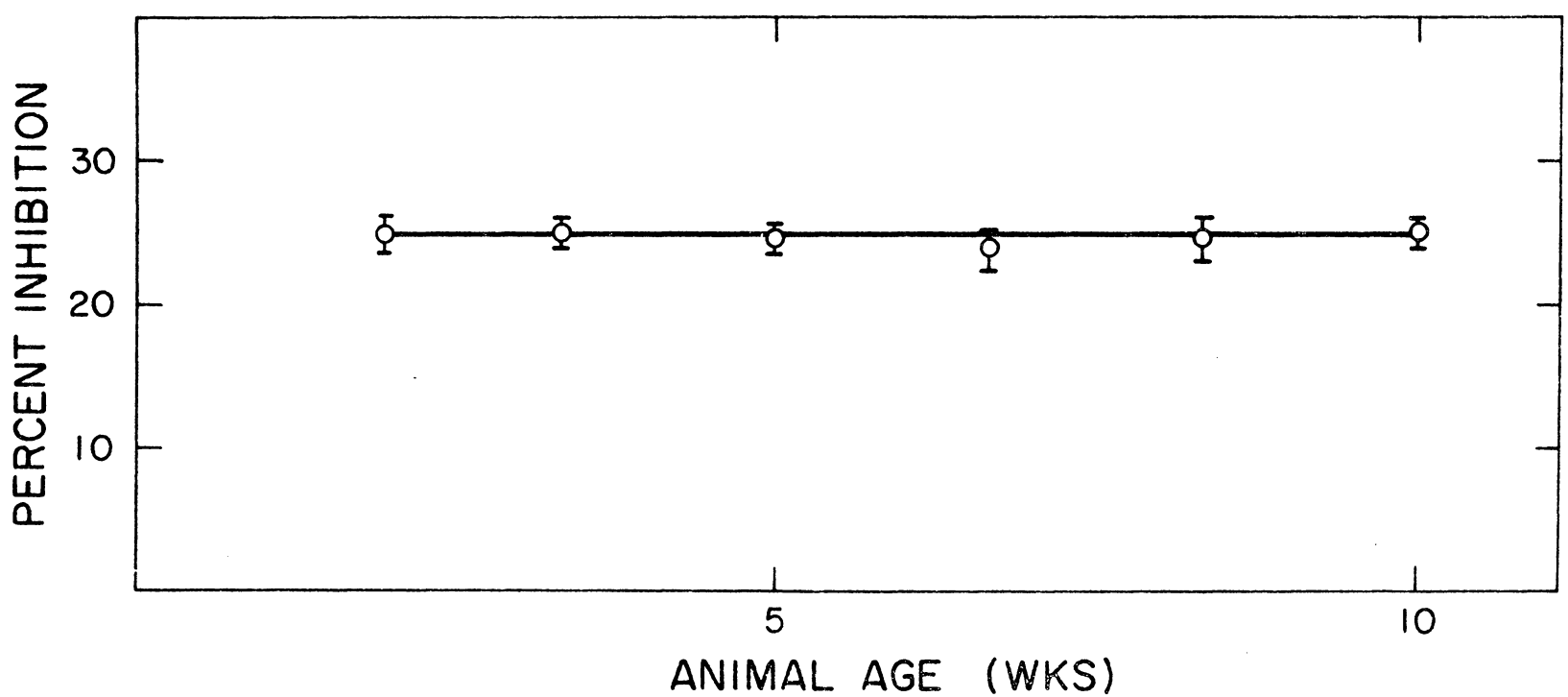
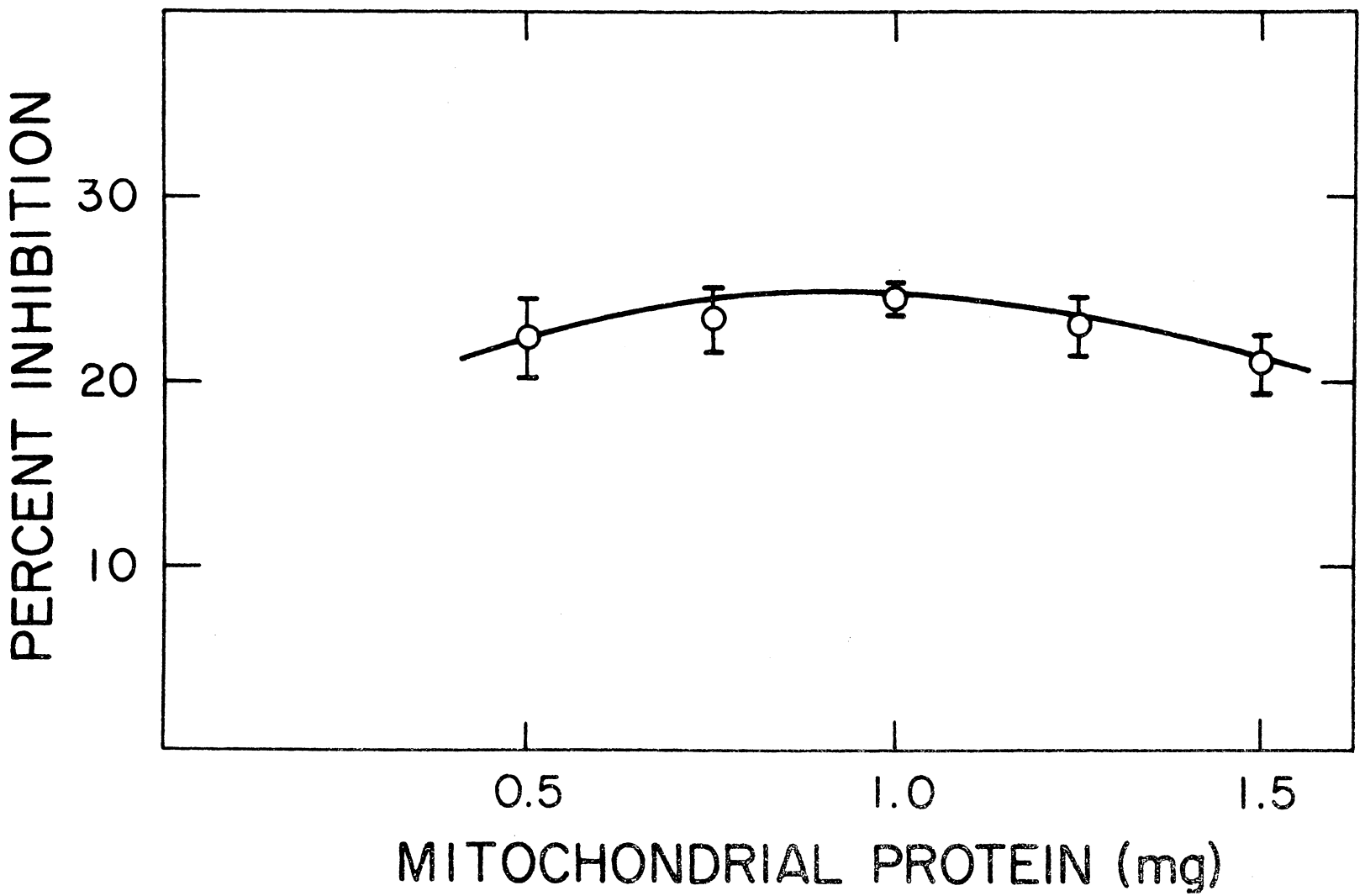


Figure 6. Inhibition of oxygen consumption as a function of mitochondrial protein concentration.

Results of 3 different experiments. AFB_1 was present at 0.25 mM. Bars indicate standard error. Succinate concentration 5 mM.



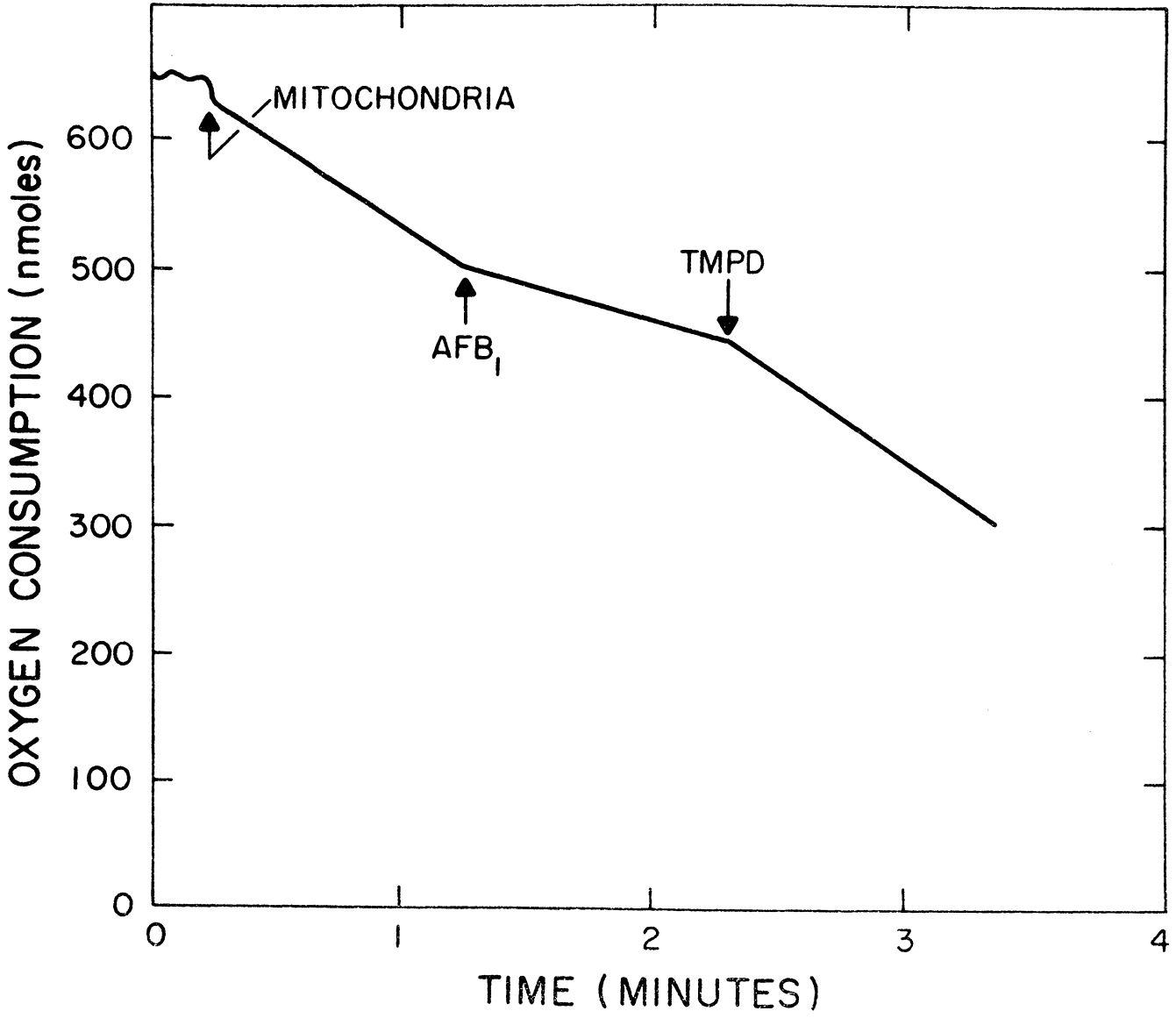
the consumption of oxygen with our instrument becomes limiting. At protein levels above 1.5 mg per 3.0 ml, the consumption of oxygen does not increase linearly. There is also the possibility that the number of AFB_1-sensitive sites begins to exceed the availability of AFB_1 molecules. Both these effects may contribute to the deviation at the upper end of the curve. All experiments were carried out at the optimal protein concentration.

The metabolism of glutamate is rather complex. A portion of the glutamate is metabolized by glutamate dehydrogenase to α-ketoglutarate and ammonia. The metabolism of α-ketoglutarate is then accomplished by the tricarboxylic acid cycle. The majority of the glutamate is metabolized by transamination. The products are aspartate and α-ketoglutarate. Aspartate is then exchanged to the medium for glutamate [126,127,128]. It could be argued that AFB_1 inhibits one of the processes involved in transamination. The metabolism of glutamate also results in the production of succinate which is metabolized by its usual route. For the reasons just discussed, glutamate is not a good NAD-dependent model substrate.

β-hydroxybutyrate is an NAD-dependent substrate that requires no ancillary preparation for dehydrogenation. The product of dehydrogenation is acetoacetate, and it is not further metabolized in liver mitochondria [129]. Fig. 7 shows the effect of 0.48 mM AFB_1 on the oxidation of β-hydroxybutyrate. As can be seen a 42% inhibition is obtained. The addition of 0.2 mM TMPD completely overcomes the inhibition by AFB_1. Indeed the rate of respiration is about 10 nmoles/min

Figure 7. Effect of N, N, N', N'-tetramethyl-p-phenylenediamine on the inhibition of β-hydroxybutyrate oxidation by aflatoxin B_1.

Representative experiment that has been repeated more than 10 times. AFB_1 concentration is 0.48 mM. TMPD concentration 0.2 mM, β-hydroxybutyrate concentration 10 mM.



faster when TMPD is present. TMPD is reduced by cytochrome b and oxidized by cytochrome c or c_1 subsequently by-passing the second site of phosphorylation. This indicates that AFB_1 inhibits at the second crossover point. The fact that the rate of inhibition after the addition of TMPD was the same as it was before the addition of AFB_1 indicates that there was no inhibition at the first or third crossover points.

Fig. 8 indicates that the oxidation of succinate is also inhibited by AFB_1, and overcome by TMPD. The rate of oxygen consumption after the addition of TMPD is the same or slightly faster than before the addition of AFB_1 again indicating that there is no inhibition at the third crossover point. In Fig. 9 the method of Sanadi and Jacobs was employed to further investigate the inhibition at the third crossover point, i.e., between cytochrome c and cytochrome oxidase [122]. No inhibition was observed in the presence of 0.48 mM AFB_1. The small blip is a potentiometric perturbation due to the DMF employed as a solvent. The rate of oxygen consumption did not change.

The data discussed above indicate that AFB_1 inhibits electron flow, but it does not indicate whether the site of inhibition is in the electron transport chain or in the phosphorylating function ancillary to the chain. Table I shows the inhibition of electron flow when DNP is used to stimulate oxygen consumption. The inhibition is greater than when ADP is used. The increase in inhibition is tentatively attributed to an alteration in mitochondrial permeability. Hemker and others have stated that among its other postulated functions DNP also has a general destructive effect on mitochondrial structure [130].

Figure 8. Effect of N, N, N', N'-tetramethyl-p-phenylenediamine on the inhibition of succinate oxidation by aflatoxin B_1.

Representative experiment that has been repeated more than 10 times. AFB_1 concentration is 0.48 mM. TMPD concentration 0.2 mM. Succinate concentration 5 mM.

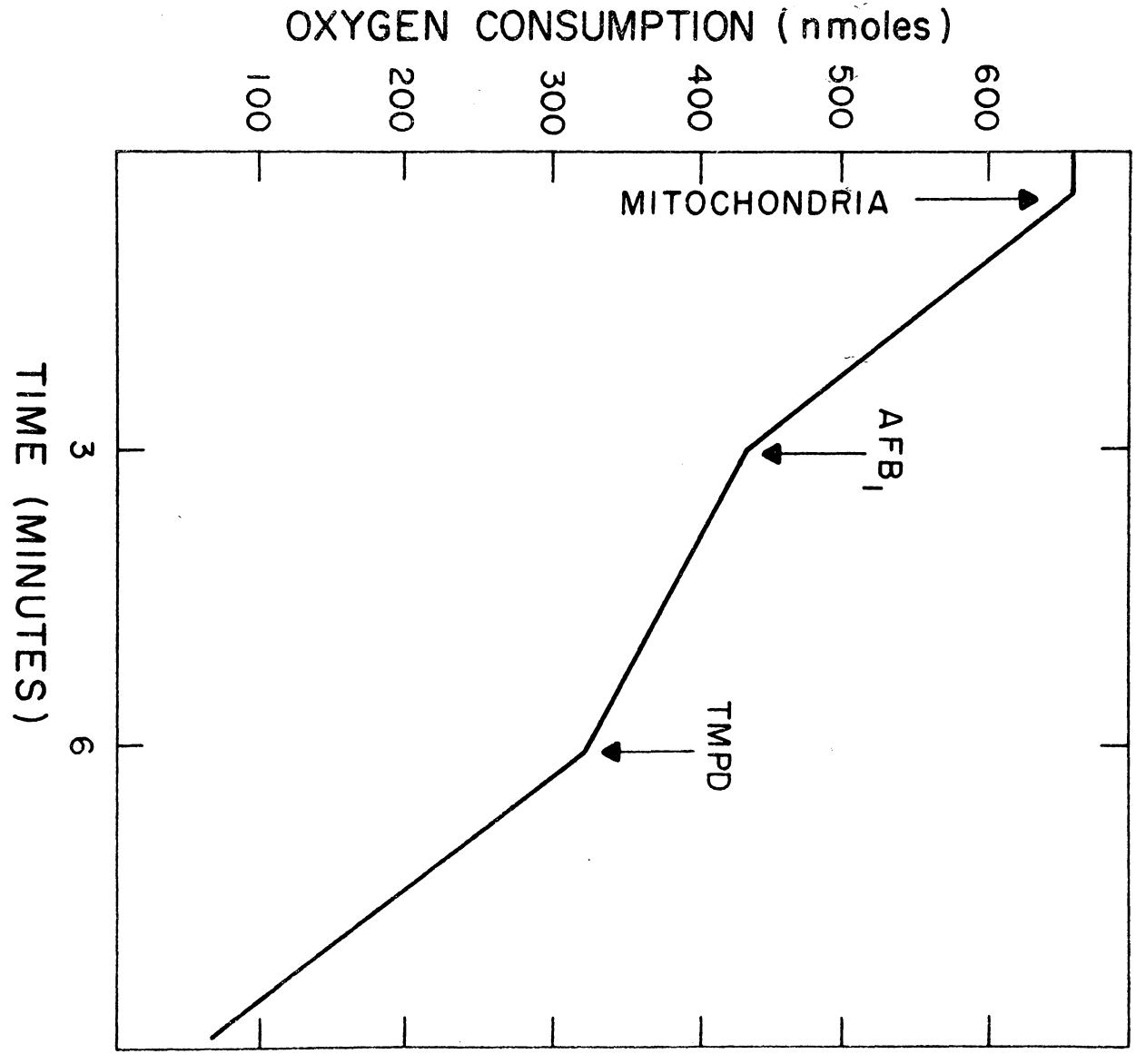


Figure 9. Effect of aflatoxin B_1 on the flow of electrons through complex IV using whole mitochondria.

Representative experiments that has been repeated 10 times. AFB_1 concentration is 0.48 mM; Ascorbate concentration 1.5 mM; TMPD concentration 1 mM.

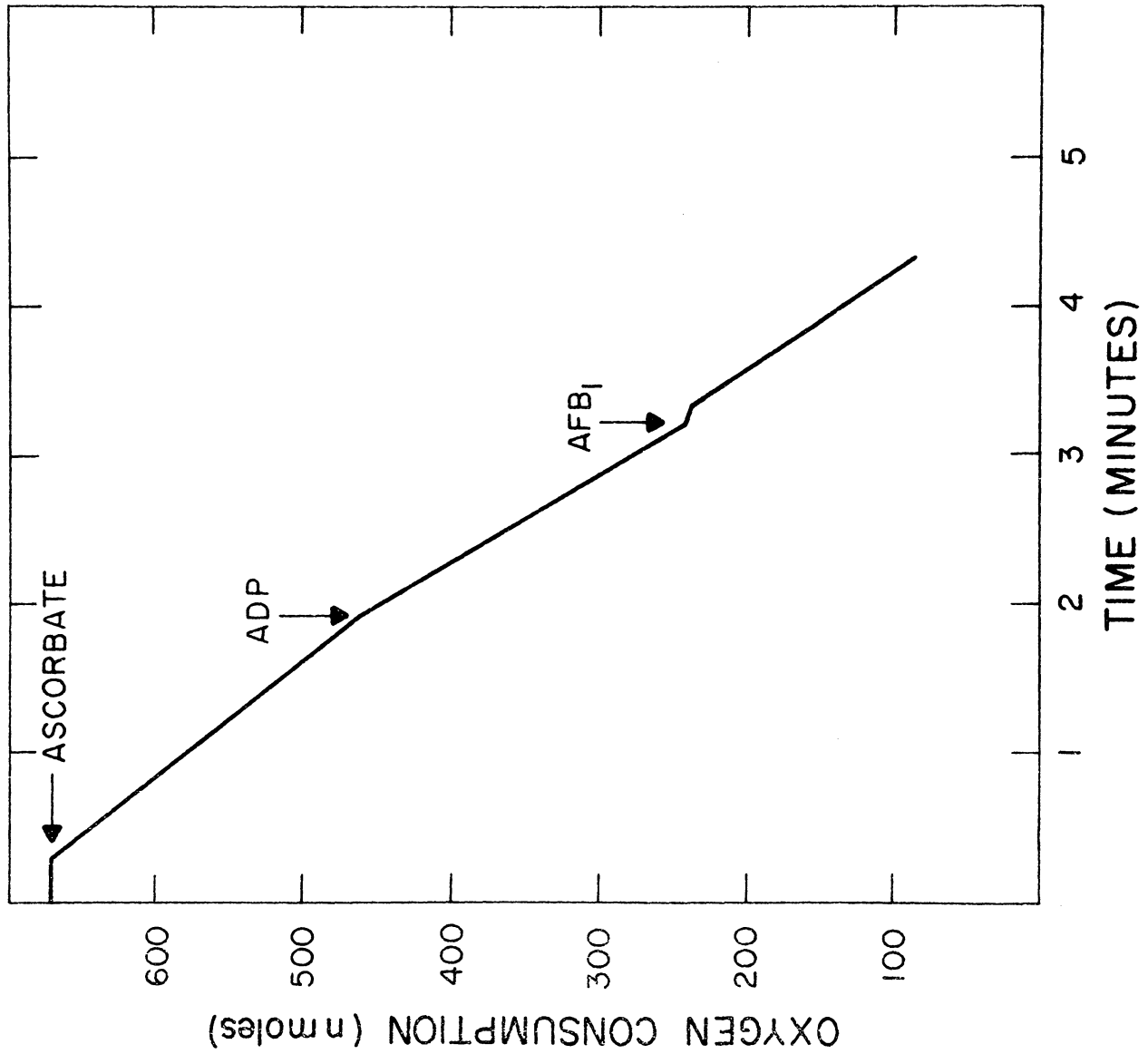


Table I. Inhibition of dinitrophenol stimulated oxygen consumption by aflatoxin B_1 using whole mitochondria from protein sufficient animals.

Aflatoxin Concentration	0.25 mM	0.32 mM	0.4 mM
DNP	33 ± 0.5%	44 ± 1.6%	63 ± 2.0%
ADP	25 ± 0.3%	29 ± 0.4%	36 ± 1.6%

Optimal DNP concentration was determined by titration to be 32 μM.

Table II shows the effect of AFB_1 on the ADP:O ratio. Ascorbate and TMPD were used to assay the third site of phosphorylation. Succinate was used to assay the second and third sites of phosphorylation. Glutamate and malate were used to assay all three sites of phosphorylation. As can be seen, there is no effect by AFB_1 even at a concentration of 0.48 mM on phosphorylation at the third site. There is however an effect on the ADP:O ratio resulting from the oxidation of succinate. There is also a marked effect on the ADP:O ratio resulting from the oxidation of glutamate and malate.

Table III shows the effect of AFB_1 on each of the three sites of phosphorylation. This was accomplished by subtracting the values obtained with a given concentration of AFB_1 from the value obtained in the untreated control. If, for example, one then wished to obtain the value for only the first site at a given concentration, the effect at the third and second sites for that concentration were then subtracted from the total effect seen on the ADP:O ratio using glutamate and malate at that concentration. The data indicate that AFB_1 has no effect on ATP synthesis at the third site. There is however a concentration-dependent effect at the second site. There is also a marked concentration-dependent effect at the first site. These values indicate that there are at least two independent ATP synthesizing systems in rat liver mitochondria. Indeed it also appears that there is a third distinctly different site, but the possibility of a co-operative effect between the sites cannot be ruled out.

In order to examine the effect of dietary protein on the inhibition

Table II. Effect of aflatoxin B_1 on the ADP:O ratio using whole mitochondria from protein sufficient animals

Malate, glutamate, and succinate concentration 5 mM.

Aflatoxin B_1 concentrate	Glutamate Malate	Succinate	Ascorbate +TMPD
DMF	2.55 ± 0.15	1.75 ± .04	.98 ± .02
0.25 mM	2.26 ± 0.02	1.63 ± .04	.98 ± .05
0.32 mM	1.91 ± 0.00	1.56 ± .00	.96 ± .08
0.4 mM	1.78 ± 0.02	1.52 ± .00	.96 ± .04
0.48 mM	1.65 ± 0.00	1.46 ± .00	.98 ± .04

Glutamate, malate, succinate, 5 mM, ascorbate, 1.5 mM; TMPD, 1 mM.

Table III. Effect of aflatoxin B_1 on the ADP:0 ratio at each site of phosphorylation computed from Table II.

Site	I	II	III
Substrate	Glutamate Malate	Succinate	Ascorbate TMPD
0.25 mM	.17	.12	.00
0.32 mM	.45	.19	.02
0.4 mM	.54	.23	.02
0.48 mM	.61	.29	.03

Values are the reduction of the ADP:0 ratio at each site due to the presence of the given concentration of AFB_1.

of mitochondrial function by AFB_1, 3 groups of weanling rats (50-60 g) were fed semipurified diets according to the regimen described in the Methods section. All animals were fed for 15 days at which time the animals were sacrificed and mitochondrial preparations were made. Group I animals were fed a 5% casein diet; Group II, a 20% casein diet pair-fed to Group I; and Group III, a 20% diet fed *ad libitum*. The per cent inhibition of state 3 mitochondria by AFB_1 are shown in Table IV. The 25% inhibition observed for the Group III animals is comparable to the inhibition of the previous animals maintained on the commercial laboratory rat chow. However, a marked reduction in the inhibition is observed in the Group I animals when compared either to Group II or Group III. The reduction of the inhibition observed for the animals in Group II compared to Group III and presumably associated with the modest deprivation of calories is much less than the reduction caused by the protein deprivation seen in Group I.

The inhibition obtained above is consistent with the results obtained when the effect of AFB_1 on the ADP:O ratio of mitochondria from protein deficient animals was examined. These data are shown in Table V. The data are dissimilar to those obtained with lab chow fed rats shown in Table II. AFB_1 in either case has no effect on the ADP:O ratio at the third site. The effect on the ADP:O ratio of mitochondria oxidizing succinate is reduced although the reduction is not concentration-dependent. The lowest concentration appears to inhibit the phosphorylation associated with the oxidation of succinate almost maximally. This is in contrast to the mitochondria from lab chow fed rats where the depression occurred

Table IV. Inhibition of ADP-stimulated oxygen consumption by aflatoxin B_1 using whole mitochondria from protein deficient animals.

Dietary Group	Respiratory Control Ratio	Per cent inhibition with AFB_1	
		0.25 mM	0.4 mM
I	5.6 ± 0.0	14.4 ± 1.3	20.2 ± 0.2
II	5.8 ± 0.0	21.0 ± 1.1	33.0 ± 2.1
III	5.7 ± 0.3	25.0 ± 0.6	36.0 ± 1.4

Table V. Effect of aflatoxin B_1 on the ADP:O ratio using whole mitochondria from protein deficient animals.

The result of triplicate determinations done on three different days.

Aflatoxin B_1 Concentrate	Glutamate Malate	Succinate	Ascorbate TMPD
DMF	2.66 ± .07	1.77 ± .04	1.01 ± 0.02
0.25 mM	2.49 ± .06	1.53 ± .02	.99 ± .05
0.32 mM	2.40 ± .05	1.48 ± .05	.99 ± .08
0.4 mM	2.36 ± .04	1.51 ± .02	.97 ± .03
0.48 mM	2.31 ± .05	1.44 ± .05	.96 ± .01

Glutamate, malate, succinate, 5 mM; ascorbate, 1.5 mM; TMPD, 1 mM.

progressively with increasing concentration. The ADP:O ratio resulting from the oxidation of glutamate and malate in mitochondria from protein deficient animals is not as sensitive to AFB_1 as it is in mitochondria from protein sufficient animals. This is attributed to the decrease in permeability of the mitochondrial inner membrane in protein deficient animals.

Table VI shows the effect of AFB_1 on each of the three sites of phosphorylation. There is no effect on phosphorylation at the third site. The effect on the second site is quantitatively similar to the mitochondria from protein sufficient animals though it is not concentration-dependent. In contrast to mitochondria from protein sufficient animals there is no effect on the first site of phosphorylation. This is attributed to the impermeability of the mitochondrial inner membrane; though there may also be changes in mitochondrial structure due to protein deficiency which decrease the effects of AFB_1 at the first site.

To examine the premise that AFB_1 is variably permeable to the mitochondrial inner membrane, Gregg particles were prepared. Fig. 10 shows schematically the preparation of Gregg particles. As can be seen, sonication removes all prospective barriers and allows direct access to the inner or M side of the inner membrane.

The solution in which Gregg particles are suspended for oxygen electrode work is different from that used for whole mitochondria in that it contains no sucrose and its ionic strength is lower. This necessitates redetermination of the CMC. Fig. 11 shows the determination of the CMC in the Gregg particle oxygen electrode solution.

Table VI. Effect of aflatoxin B_1 at each site of phosphorylation computed from Table III.

	SITE I	SITE II	SITE III
Substrate	Glutamate Malate	Succinate	Ascorbate TMPD
0.25 mM	0.00	.22	.02
0.32 mM	.00	.27	.02
0.4 mM	.04	.22	.04
0.48 mM	.00	.28	.05

Values are the reduction of the ADP:O ratio at each site due to the presence of the given concentration of AFB_1.

Figure 10. Schematic representation of the production of Gregg particles by sonication [42].

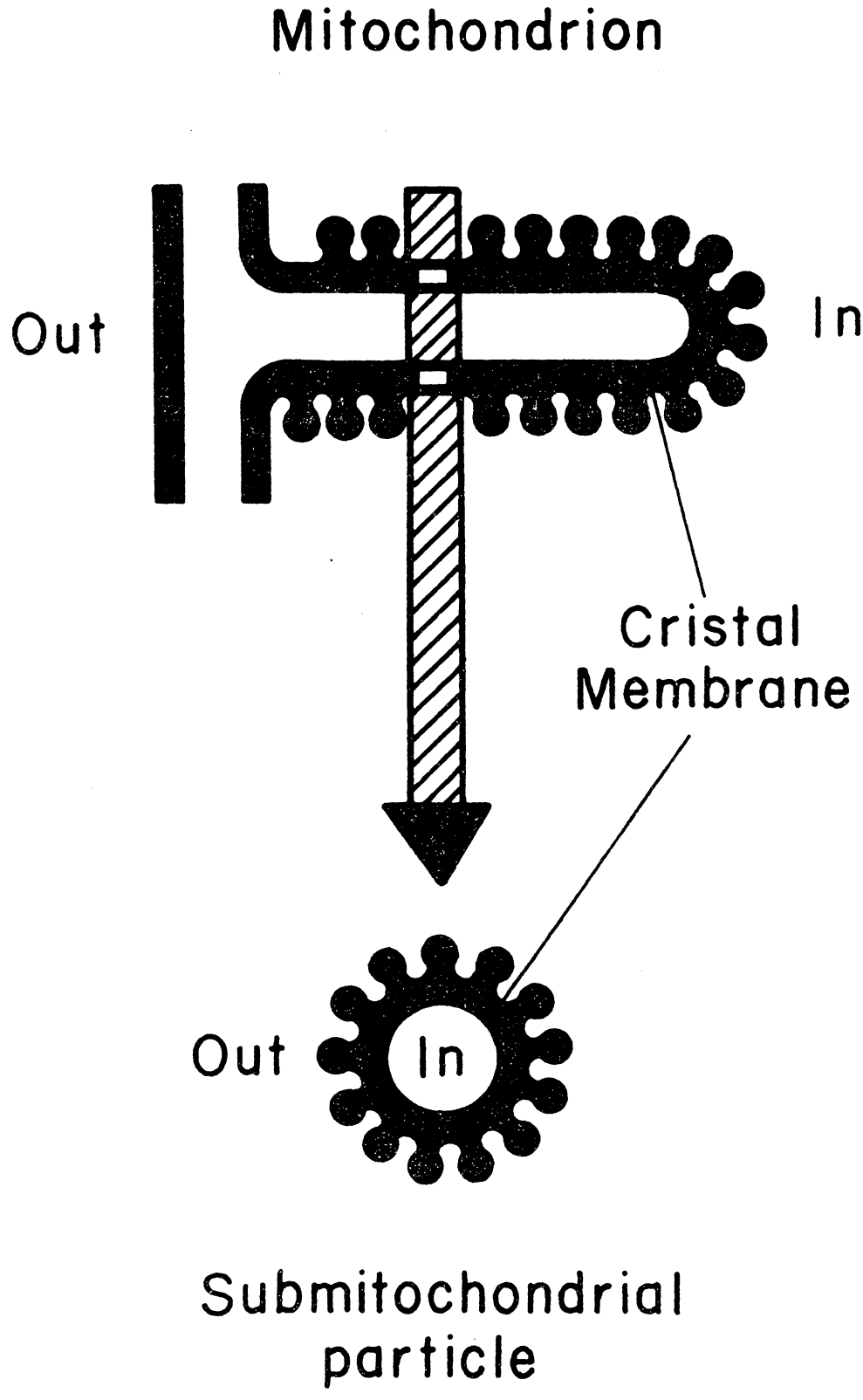
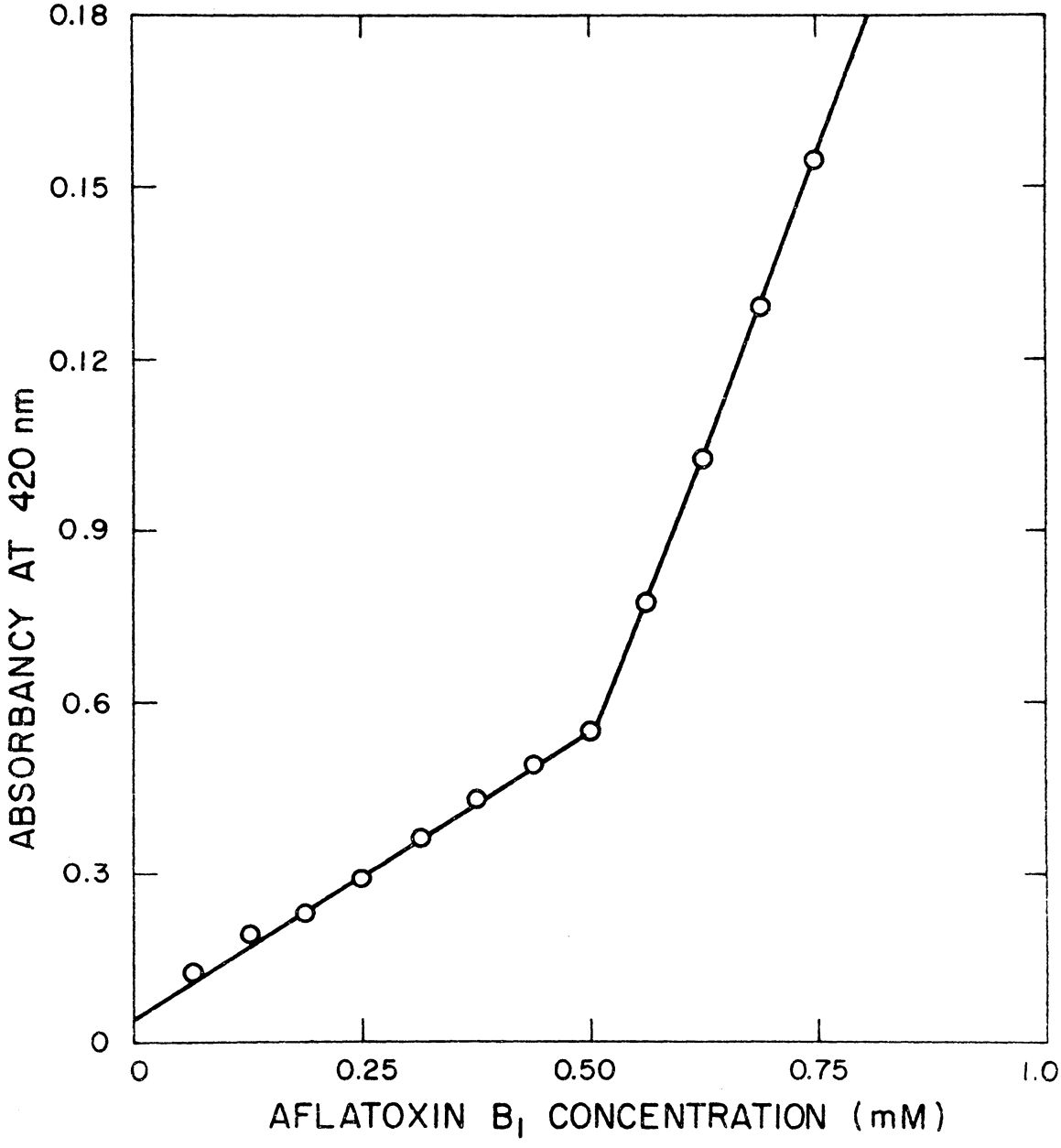


Figure 11. Determination of the critical micelle concentration under the conditions employed with Gregg particles.

Repeated 3 times.



The CMC is 0.53 mM under these conditions. The increase in the CMC can be attributed to the increase in water availability in the Gregg particle solution. There is no sucrose present to tie up water in solvation spheres. The decrease in ionic strength would also mean fewer hydration spheres and more water available to accommodate AFB_1.

Fig. 12 shows the effect of AFB_1 on the oxidation of β-hydroxybutyrate by Gregg particles. As can be seen, a 63% inhibition is obtained with 0.53 mM AFB_1. The addition of menadione at a concentration of 38 μM had no effect on the inhibition. Menadione bridges the inhibition by rotenone and amytal at the first site of phosphorylation [131]. Menadione has no effect on the inhibition by AFB_1 indicating that it does not affect the first crossover point. The removal of barriers to penetrability should expose the first site to the maximal concentration of AFB_1. The data indicate that the first crossover point is not sensitive even at maximal concentrations of AFB_1.

Fig. 13 shows the inhibition by AFB_1 (0.53 mM) on the oxidation of β-hydroxybutyrate by Gregg particles. The addition of TMPD completely overcomes the inhibition. Indeed it is about 20 nmoles per min faster. This indicates that AFB_1 does not inhibit at the first or third crossover points. If it did, the rate of respiration could not be restored. The degree of inhibition obtained with Gregg particles in the presence of 4 mM amytal, 4 μg of antimycin, and 1 mM sodium cyanide is 54%, 64% and 50%, respectively [44]. This indicates there is considerable inhibitor insensitive respiration in Gregg particles. In these experiments it amounts to 36% of the total respiration with AFB_1 and 30% with antimycin.

Figure 12. Effect of menadione on the inhibition of β-hydroxybutyrate oxidation by aflatoxin B_1 using Gregg particles.

Representative experiment that has been repeated more than 10 times on 4 different preparations. AFB_1 concentration 0.53 mM; menadione 38 µm; approximately 0.1 mg particle protein employed. β-hydroxybutyrate concentration 10 mM.

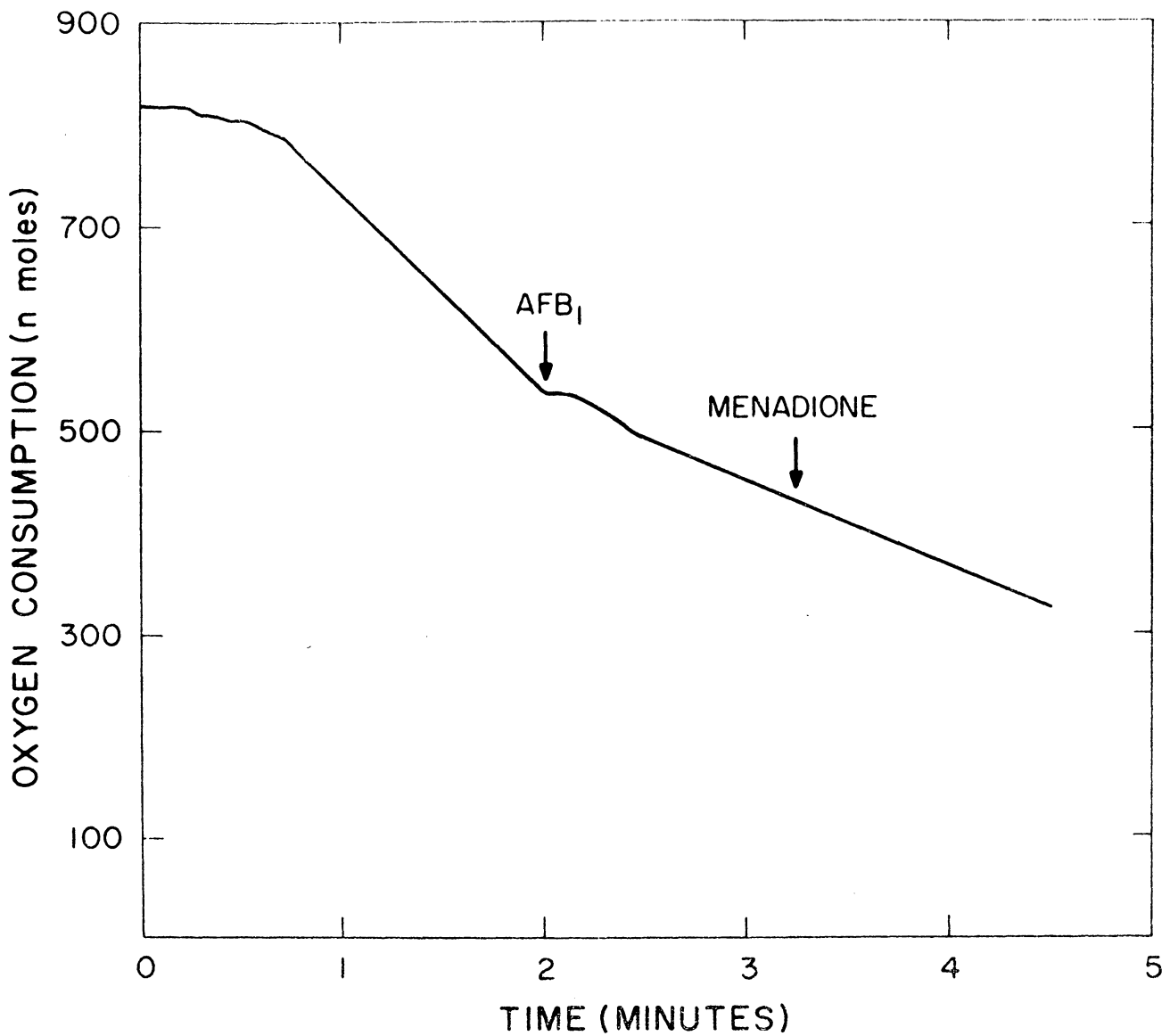
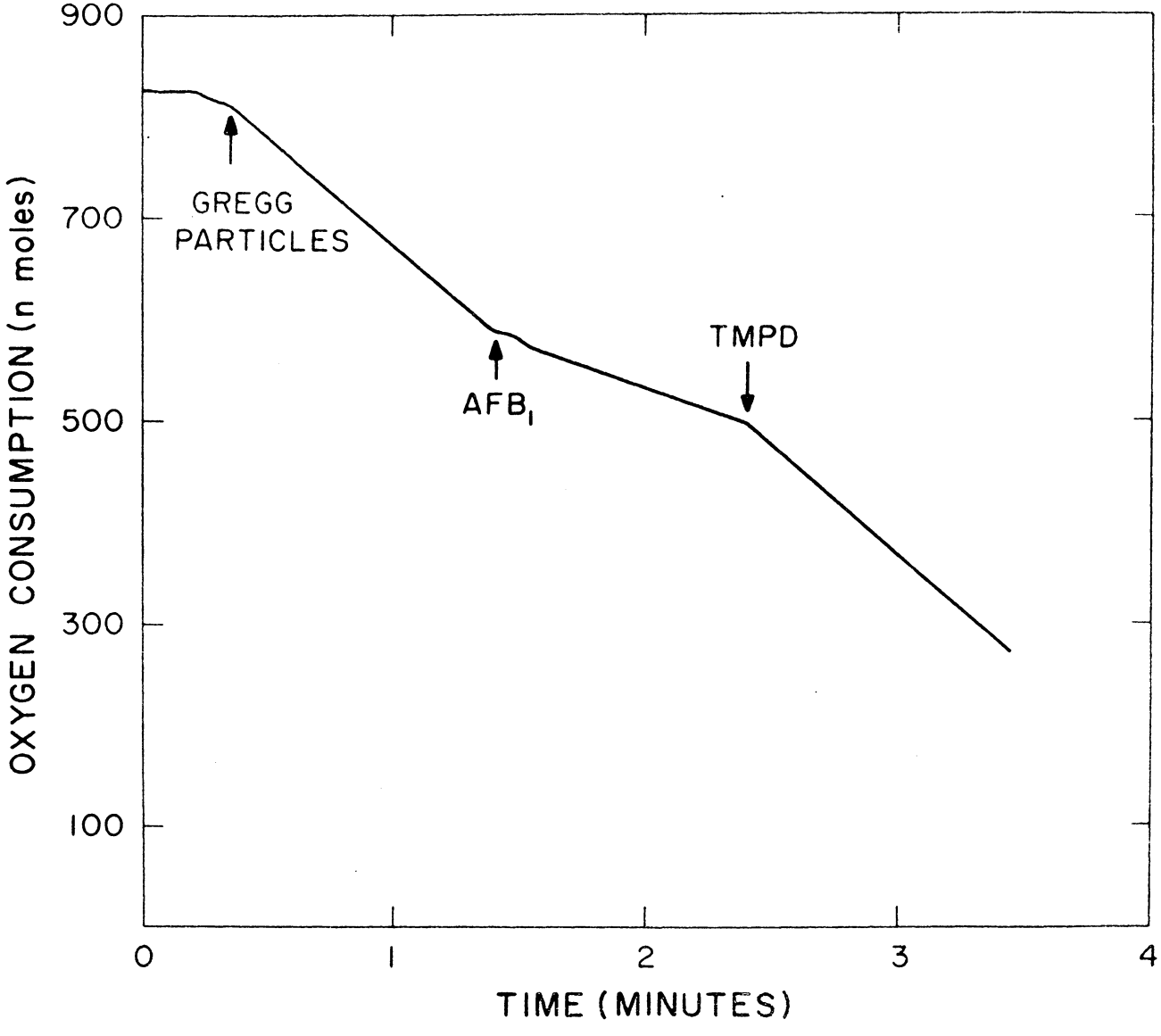


Figure 13. Effect of N, N, N', N'-tetramethyl-p-phenylenediamine on the inhibition of β-hydroxybutyrate oxidation by aflatoxin B_1 using Gregg particles.

Representative experiment that has been repeated more than 10 times on 4 different preparations. AFB_1 concentration 0.53 mM; TMPD concentration 0.2 mM; NAD concentration 0.2 mM; β-hydroxybutyrate concentration 10 mM; approximately 0.1 mg particle protein employed.



This is similar to the values obtained by Gregg shown above.

Fig. 14 shows the effect of AFB_1 on the oxidation of succinate by Gregg particles. The oxidation of succinate does not proceed as rapidly as that of β-hydroxybutyrate. This coupled with the inhibitor insensitive respiration makes working with succinate and Gregg particles difficult. As can be seen in Fig. 14, TMPD restores the inhibitor-sensitive respiration completely with 10 to 20 nmoles per min additional respiration, as is indicated in the literature [121]. This indicates that the site of inhibition is between cytochrome b and cytochromes c or c_1. The 0.2 mM NAD present in the medium for oxidizing β-hydroxybutyrate is a possible source of error. The fact that the same results can be obtained with both β-hydroxybutyrate and succinate indicates that the contribution from direct oxidation of NADH is minimal [132].

In Fig. 15 the procedure of Sanadi and Jacobs was used to examine the inhibition by AFB_1 at the third crossover point. AFB_1 at a concentration of 0.53 mM has no effect on the flow of electrons through the third crossover point. Ascorbate (1.5 mM) and TMPD (1 mM) were used to specifically route electrons through complex IV. The blip in the tracing is due to a potentiometric perturbation caused by the DMF employed as a solvent for AFB_1.

Fig. 16 shows the inhibition of oxygen consumption by Gregg particles from protein sufficient animals, expressed as a per cent of control, as a function of the AFB_1 concentration. The curve is hyperbolic in nature. The maximum inhibition obtained is 63%. This limit is due to the large amount of inhibitor insensitive respiration

Figure 14. Effect of N, N, N', N'-tetramethyl-p-phenylenediamine on the inhibition of succinate oxidation by aflatoxin B_1 using Gregg particles.

Representative experiment that has been repeated more than 10 times on 4 different preparations. AFB_1 concentration 0.53 mM; TMPD concentration 0.2 mM; succinate concentration 10 mM; approximately 0.1 mg particle protein employed.

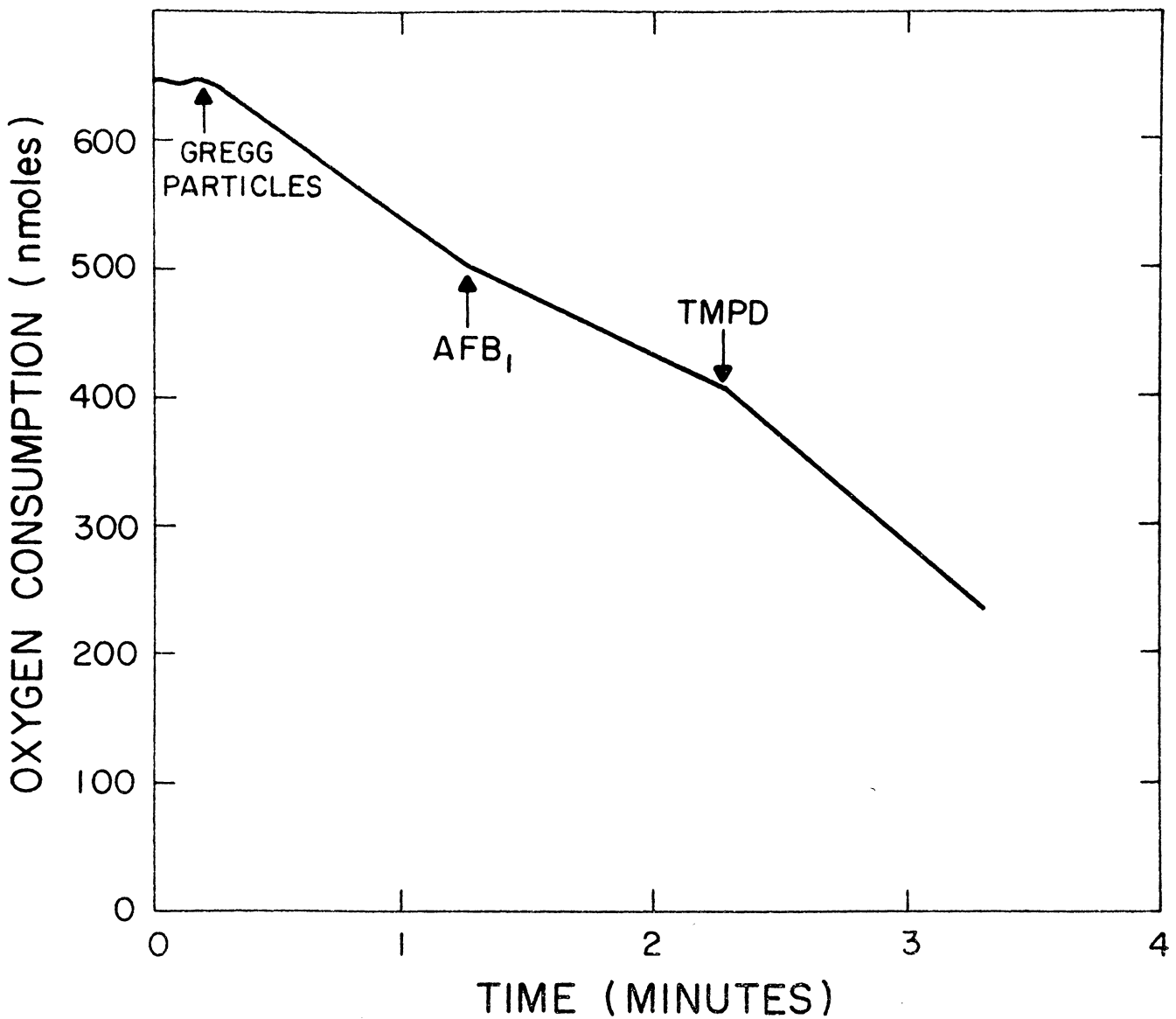


Figure 15. Effect of aflatoxin B_1 on the flow of electrons through complex IV using Gregg particles.

Representative experiment that has been repeated more than 5 times on 3 different preparations. Ascorbate concentration 1.5 mM; TMPD concentration 1 mM; AFB_1 concentration 0.53 mM; approximately 0.1 mg particle protein employed.

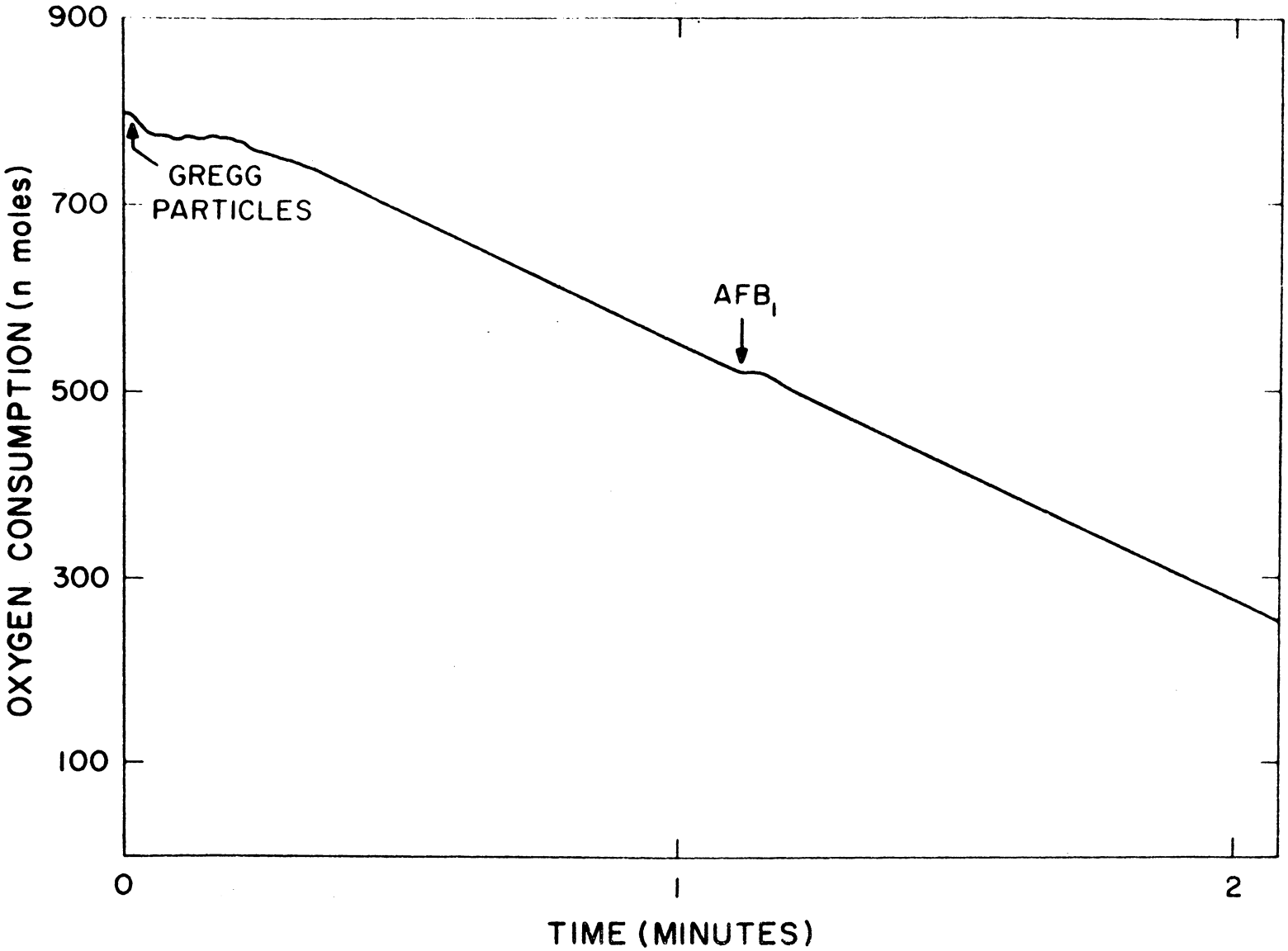


Figure 16. Inhibition of oxygen consumption as a function of the aflatoxin B_1 concentration using Gregg particles from protein sufficient animals.

This experiment has been replicated on 3 preparations; approximately 0.1 mg particle protein employed. β-hydroxybutyrate concentration 10 mM.

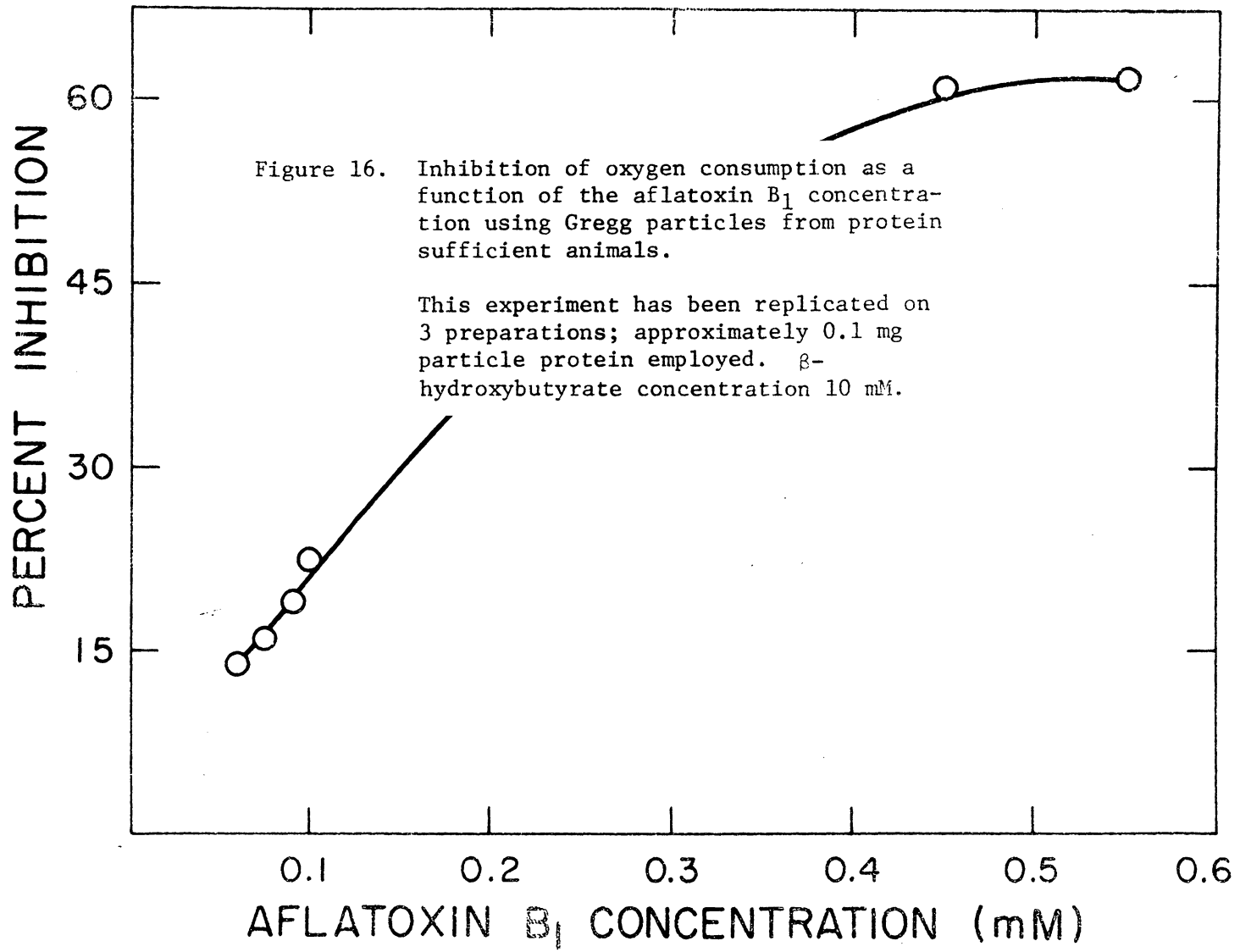


Figure 16. Inhibition of oxygen consumption as a function of the aflatoxin B_1 concentration using Gregg particles from protein sufficient animals.

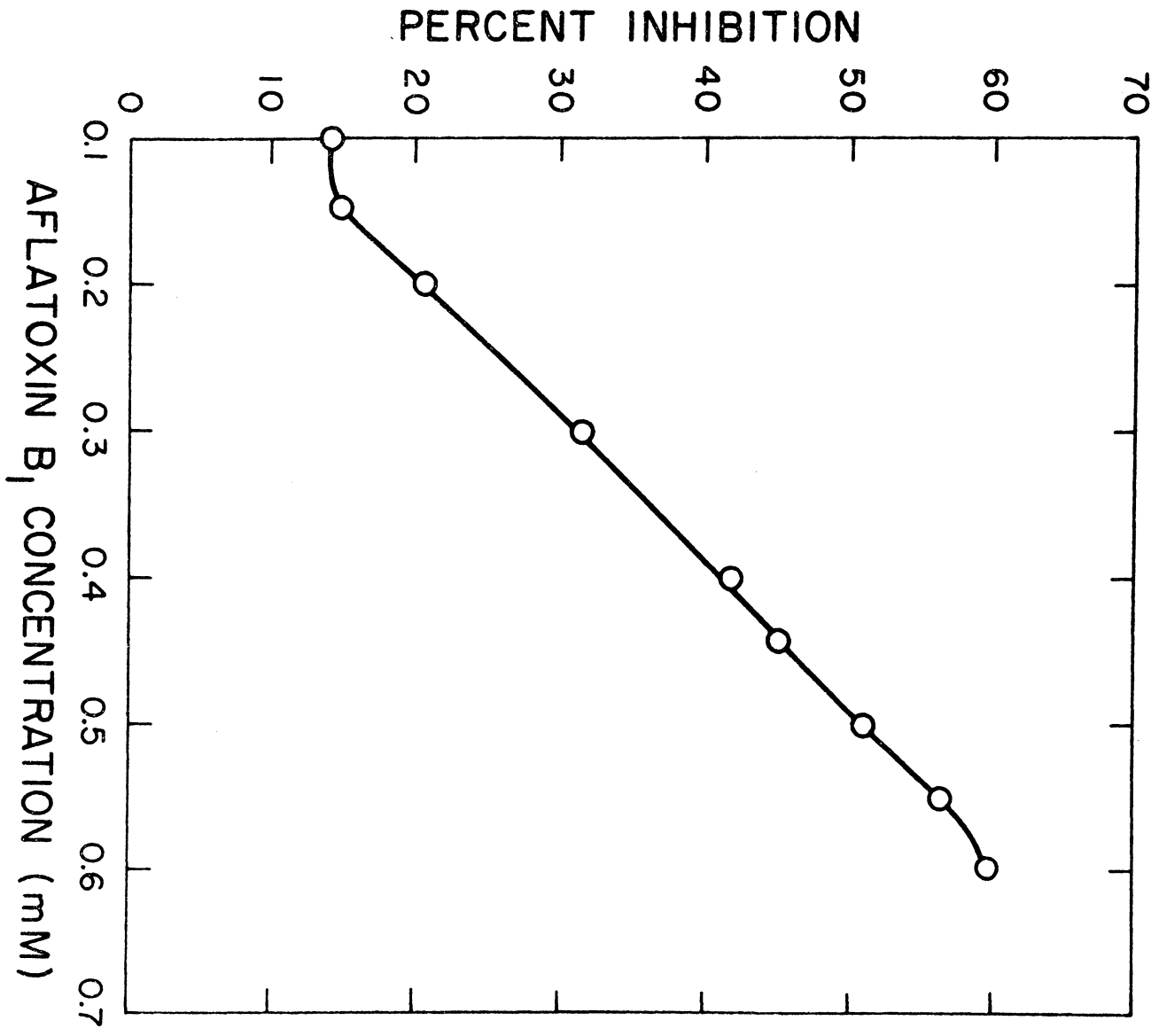
This experiment has been replicated on 3 preparations; approximately 0.1 mg particle protein employed. β-hydroxybutyrate concentration 10 mM.

previously discussed. The hyperbolic nature of the inhibition curve with submitochondrial particles is reminiscent of the effects of AFB_1 on whole mitochondria. It would appear that there is one site in complex III to which AFB_1 binds. This is in contrast to antimycin which inhibits at a similar site but which has a sigmoidal inhibition curve. These two inhibitors probably bind to different proteins in the complex. The inhibition is probably due to a conformational change altering the physical juxtaposition of cytochrome b and cytochrome c_1. Conformational changes in complex III are well known [133,134].

Fig. 17 shows the inhibition of oxygen consumption, expressed as a per cent of control, as a function of AFB_1 concentration for Gregg particles prepared from rats fed a 5% protein diet. The curve is again sigmoidal in nature. The shape of the curve is somewhat different from the curve shown in Fig. 16 for protein sufficient animals. This is presumably due to protein deprivation. When oxidizing β-hydroxybutyrate, added cytochrome c will not stimulate respiration with these particles. This is in contrast to the behavior of Gregg particles isolated from protein sufficient animals. With these particles the addition of cytochrome c stimulates oxygen consumption. Presumably the rate limiting step in the particles from the protein deficient animals is elsewhere in the respiratory chain. β-hydroxybutyrate dehydrogenase would be a good candidate for the rate limiting step. The Km for NAD and β-hydroxybutyrate appears to be altered. This is indicated by the fact that 0.1 M NAD and 5 mM β-hydroxybutyrate will not saturate the system.

Figure 17. Inhibition of oxygen consumption as a function of the aflatoxin B_1 concentration using Gregg particles from protein deficient animals.

Each point represents an average of triplicates on 3 different preparations; approximately 0.1 mg particle protein employed. β-hydroxybutyrate concentration 10 mM.



On the other hand, these concentrations are adequate for saturation in particles from protein sufficient animals. The effect of protein deficiency on the inner mitochondrial membrane merits further study.

Thiamine deficient animals were considered a good model for evaluating the permeability of AFB_1 to whole mitochondria. Thiamine is singularly important in the economy of the mitochondrion. It is part of the pyruvate decarboxylase complex as well as the α-ketoglutarate decarboxylase complex. Thiamine deficiency results in energy-deficient mitochondria which are swollen as compared to energy-sufficient mitochondria [135]. Fig. 18 shows the effect of AFB_1 at various concentrations on oxygen consumption, expressed as a per cent of control. These are whole mitochondria with a respiratory control ratio of 4.0 when succinate was used as a substrate. The inhibition curve is clearly sigmoidal in nature. The resemblance to the curves obtained with submitochondrial particles is obvious. The maximum is in this case limited by the formation of micelles at the CMC. The inhibition by AFB_1 at the various concentrations is not as great as it is with submitochondrial particles. This could be due to restrictions in available concentrations of AFB_1 at its site of action due to permeability or the non-specific binding of AFB_1 to membranes and proteins. These mitochondria are clearly more sensitive to AFB_1 at lower concentrations. Great care had to be taken with these mitochondria due to their instability. They could only be used reproducibly for 45 min after isolation.

Fig. 19 shows the pH dependence of Gregg particles from animals fed a complete diet. The interaction between AFB_1 and its site of

Figure 18. Inhibition of oxygen consumption as a function of the aflatoxin B_1 concentration using whole mitochondria isolated from thiamine deficient animals.

Each point represents duplicate replications on 3 preparations made from 5 rats; 1 mg of mitochondrial protein was employed. Succinate concentration 5 mM.

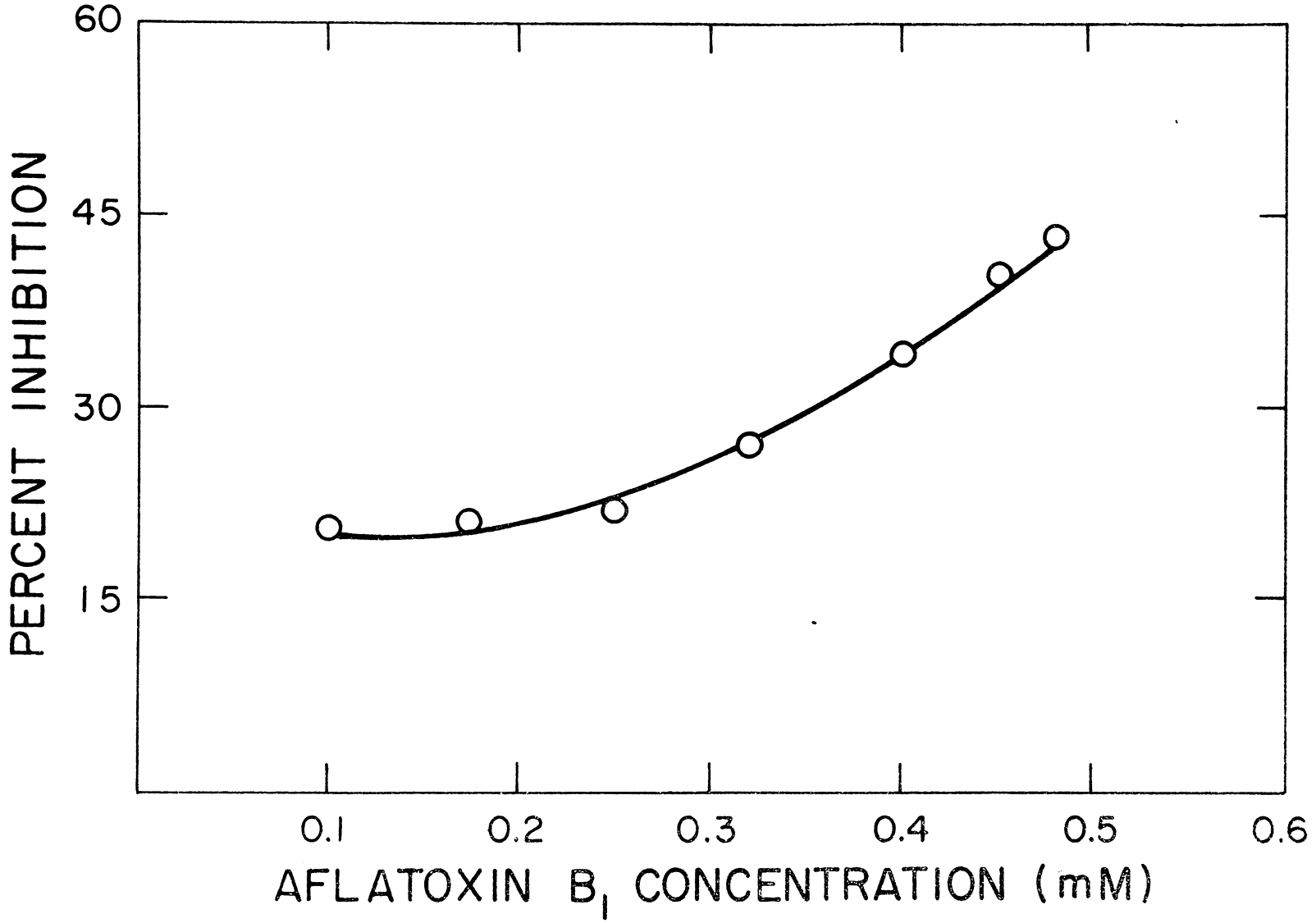
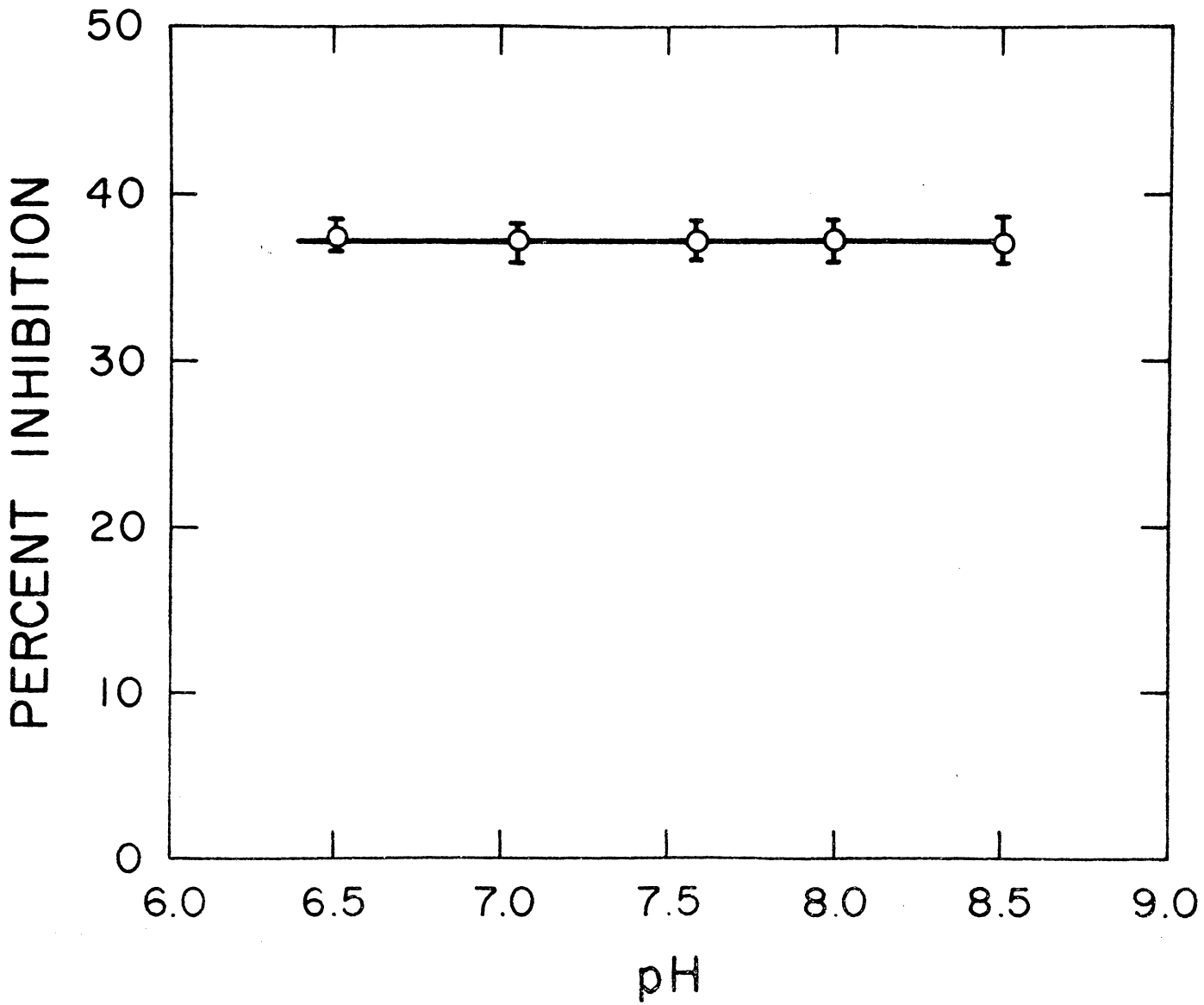


Figure 19. pH dependence of the inhibition of oxygen consumption by aflatoxin B_1 using Gregg particles.

Each point represents duplicates on 3 preparations; 0.1 mg of particle protein; aflatoxin concentration 0.25 mM. β-hydroxybutyrate concentration 10 mM.



action appears to be pH independent over the range studied. This is not surprising since there are no ionic groups on AFB_1 to influence its binding. The site of binding is very likely a hydrophobic protein, a basic protein, or a lipoprotein complex.

The data shown in Fig. 20 schematically represents the data obtained in an experiment examining spectrophotometrically the oxidation-reduction level of cytochromes b and c. The data indicate that the reduction levels of cytochrome b increased and cytochrome c decreased when 0.4 mM AFB_1 is added to Gregg particles actively oxidizing β-hydroxybutyrate. Care was taken to insure a high oxygen concentration during the course of the experiment. This experiment indicates that the point of inhibition is between cytochromes b and c. The fact that cytochrome c does not drop as much as cytochrome b can be attributed to the fact that cytochrome b is measured at its gamma peak which has a greater extinction coefficient than the alpha peak at which cytochrome c is measured. It must also be considered that complex III contains two molecules of cytochrome b and one molecule of cytochrome c_1. Cytochrome c has been shown to be rate-limiting in the oxidation of β-hydroxybutyrate; and since cytochrome c is easily lost, it is therefore likely that the structurally bound cytochrome c_1 is the predominant species contributing to the alpha peak of the cytochromes c. The block most likely occurs between cytochrome b and cytochrome c_1.

Table VII shows the effect of AFB_1 on the DNP-stimulated ATPase activity in Gregg particles. DNP-stimulated ATPase activity in Gregg particles as well as in whole mitochondria is thought to be the ATPase

Figure 20. Spectrophotometric determination of the oxidation-reduction levels of cytochromes b and c when aflatoxin B_1 is added to Gregg particles oxidizing β-hydroxybutyrate.

Repeated 5 times on 2 different preparations.

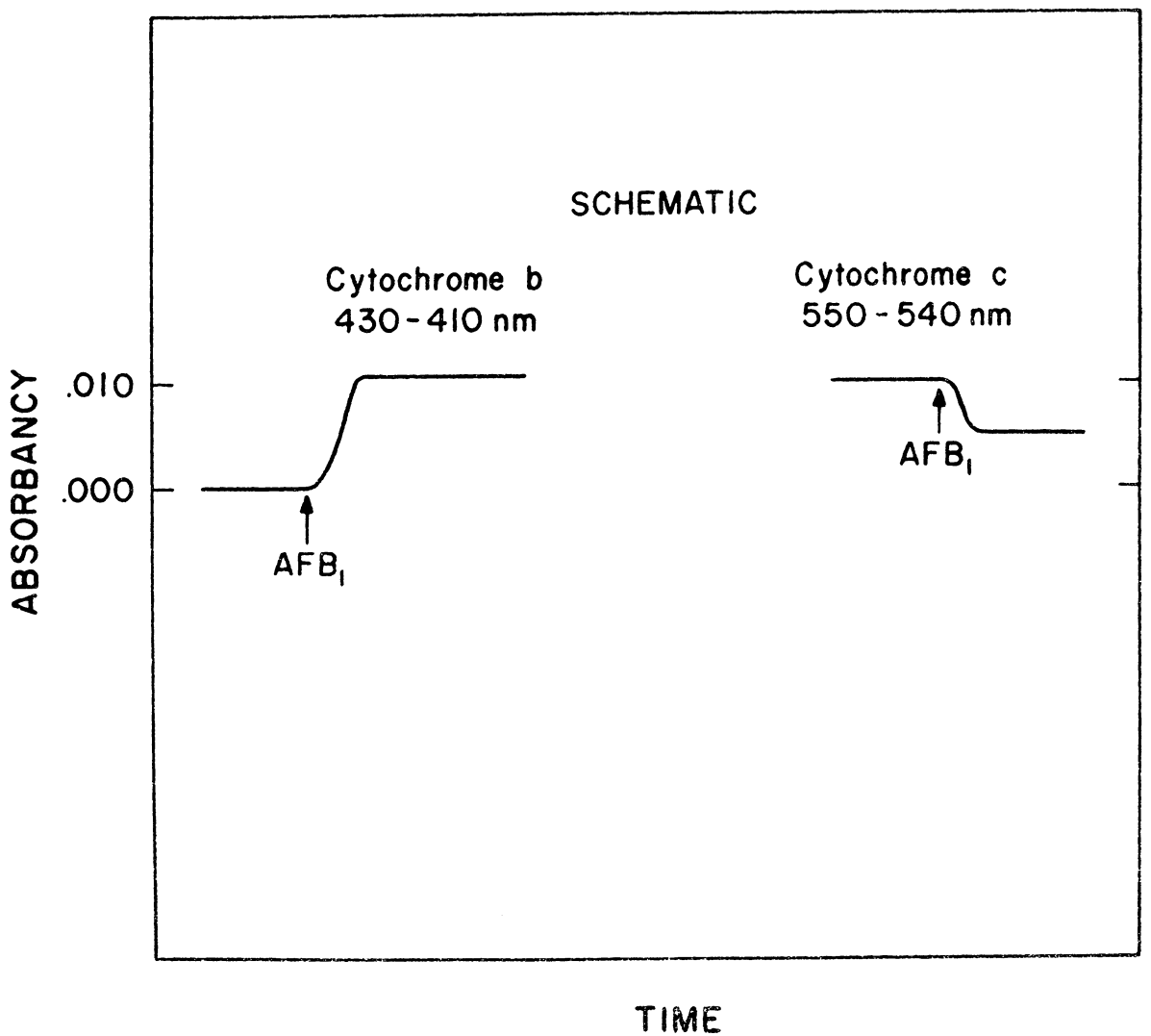


Table VII. Inhibition of dinitrophenol stimulated ATPase activity in Gregg particles.

Aflatoxin B_1 concentration 0.4 mM
Antimycin concentration 2 µg/ml

Substrate	Inhibitor	-ΔATP (mmoles)
_____	_____	3.36 ± .01
_____	Dinitrophenol	4.40 ± .02
_____	Dinitrophenol + aflatoxin B_1	4.36 ± .00
_____	Dinitrophenol + antimycin A	4.60 ± .00
β-Hydroxybutyrate + NAD^+ + succinate	Dinitrophenol + aflatoxin B_1	3.49 ± .02
β-Hydroxybutyrate + NAD^+ + succinate	Dinitrophenol + antimycin A	3.40 ± .00

activity associated with the ATP synthesizing machinery driven by the electron transport system. Abatement of this activity occurs when the electron transport system is completely reduced. In Gregg particles, this requires the addition of β-hydroxybutyrate and succinate plus an inhibitor of the electron transport system. The data indicate that the block of electron flow by 0.4 mM AFB_1 is about as good as that of 4 μg antimycin. This indicates that AFB_1 is indeed an inhibitor of electron flow and that the inhibition is indeed in the electron transport chain. The fact that ATPase activity is not inhibited in the absence of reducing substrate supports this contention.

GENERAL DISCUSSION

The results previously shown indicate that there is no inhibition, within error, i.e., 10 nmoles per min, at the first and the third crossover points in mitochondria and submitochondrial particles. The concentration of menadione employed in Fig. 12 is adequate to overcome any inhibition at the first crossover point, and no increase in rate is observed. Figs. 7 and 13 indicate that with NAD-dependent substrates the addition of TMPD completely restores the original rate of oxygen consumption. This would not be possible if there were an inhibition above cytochrome b in the electron transport system. Figs. 9 and 15 indicate that there is also no inhibition in the cytochrome oxidase portion of the electron transport chain. This is also implicit in Figs. 7, 8, 13, and 14. An inhibition in this portion of the chain would not allow TMPD to restore electron flow after inhibition by AFB_1.

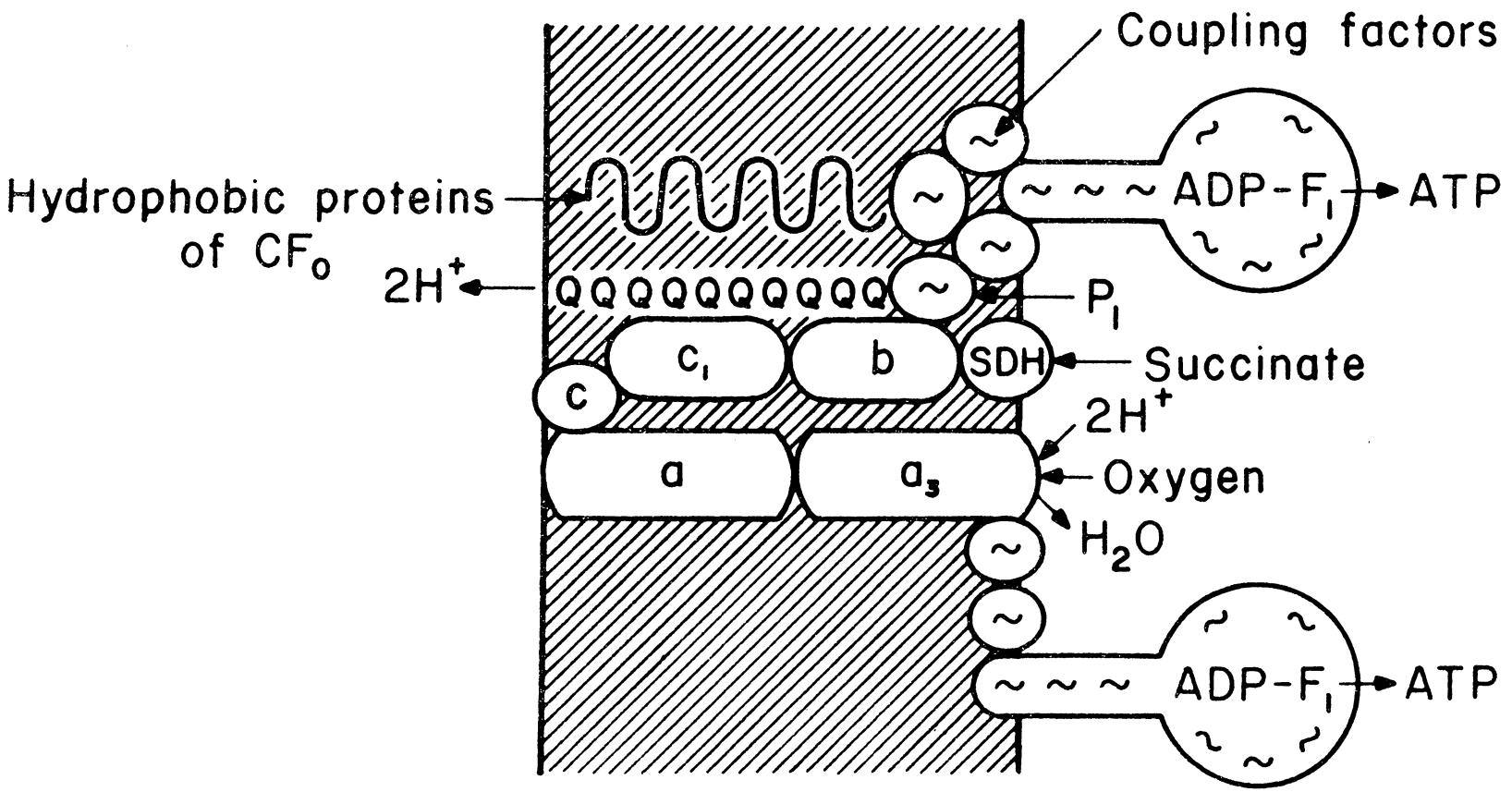
The data obtained through the use of TMPD indicate that the site of inhibition of oxygen consumption by AFB_1 is between cytochrome b and cytochromes c or c_1. The work with DNP indicates that the site of inhibition is not in the phosphorylating function ancillary to the electron transport chain. If it were in the phosphorylating function, there would be no inhibition in the presence of DNP.

The spectrophotometric data using submitochondrial particles also indicate that the site of inhibition is between cytochrome b and cytochromes c or c_1. The fact that cytochrome c is easily lost even from intact mitochondria is well known [136]. The process of sonication

is quite drastic and the evidence indicates that adequate cytochrome c is not present to maintain optimal electron transport. It is therefore probable that the major contributor to the alpha peak of cytochrome c in these studies is cytochrome c_1. The site of action of AFB_1 is on this basis more likely to be between cytochrome b and cytochrome c_1. The nature of the mitochondrial intermembrane is such that binding to either or both sides of the membrane might inhibit the flow of electrons at the second crossover point.

The inhibition of electron flow by AFB_1 is extremely complex and apparently involves binding at two different sites. This may be required to explain the sigmoidal inhibition curve obtained with thiamine deficient mitochondria and submitochondrial particles. The sites of inhibition appear to be on the M side of the inner membrane. The schematic representation of the inner membrane is shown in Fig. 21. If they were on the C side of the inner membrane, the inhibition by AFB_1 would be reduced in submitochondrial particles, not increased. The curve obtained with thiamine deficient mitochondria shown in Fig. 18 is similar to the curve shown in Fig. 4 between 0.25 and 0.48 mM. The thiamine deficient mitochondria however show a greater sensitivity to AFB_1 at lower concentrations. This is presumably due to the increased permeability of the mitochondrial inner membrane, though an alteration of the binding sites cannot be ruled out. The very steep hyperbolic curve obtained with submitochondrial particles from protein sufficient animals shown in Fig. 16 is quite different from the curves obtained with whole mitochondria. This is probably due to the increased amount

Figure 21. Schematic representation of the inner mitochondrial membrane [51].



of AFB_1 free to act as an inhibitor. There is no reduction in the effective concentration due to nonspecific binding to proteins and to other portions of the mitochondrial membranes, and there are no penetrability barriers as there would be with whole mitochondria. An alternative explanation would involve another site available for the binding of AFB_1. The binding at this site would have to increase the affinity of the other site for aflatoxin B_1 causing a greater inhibition. The possibility of structural alterations occurring during the sonication process or because the membrane is arranged in a different manner is very real. The fact that the membrane is curved with the head pieces pointing out in submitochondria particles may expose another site for binding. Complex III is a complicated array of proteins and many of its protomers could have binding sites.

Conformational changes associated with electron transport through complex III were proposed by Hatefi [133]. Two electrons go into the complex; one goes to cytochrome b and a second, possibly from coenzyme Q, is fed into the system to reduce another component in the complex which is as yet undefined. Upon addition of the second electron (also possibly a proton), there is a conformational change which brings cytochrome b into contact with cytochrome c_1. There is a possibility that this may be mediated by a nonheme iron molecule. In the absence of cytochrome c, conformational equilibrium is maintained at this point. Added cytochrome c causes cytochrome c_1 to be oxidized and there is another change in conformation back to its original position. It appears as though AFB_1 prevents the change in conformation allowing

cytochromes b and c_1 to come into position to effect the transfer of electrons.

The inhibition by AFB_1 is not sensitive to pH from 6.5 to 8.5, and this might indicate a hydrophobic mode of binding between AFB_1 and complex III or a binding involving ions uninfluenced in this pH range. If ions were involved, they would have to be of the basic variety such as, the guanidine group of arginine and the epsilon amino group of lysine. These groups have been shown to have a high affinity for the electrondense regions of AFB_1 as in its interaction with histones. These groups would also remain in the same state of ionization throughout the pH range investigated. Complex III has been shown by Hatifi to contain a highly basic protein of this type [133]. It is on the surface in contact with cytochrome c_1. This highly basic protein is low in molecular weight, soluble in acidic methanol and is purified along with cytochrome c_1. This protein would be a good candidate for one or both of the binding sites interacting with AFB_1.

The depression of the ADP:O ratio also could reasonably be expected to require penetration of the inner mitochondrial membrane. This is indicated by the fact that the phosphorylation mechanism is located at the M side of the inner membrane. The fact that depression of the ADP:O ratio and the degree of inhibition of electron flow seem to parallel each other in protein sufficient and protein deficient animals points to permeability being the limiting factor. The mechanism for the depression of the ADP:O ratio is difficult to explain. The mechanism of phosphorylation itself is not very well understood. One can only

speculate as to the function of AFB_1 but it probably involves the conformational changes associated with the inhibition of electron flow.

The inhibition of oxygen consumption obtained with 0.48 mM AFB_1 is 43%. The phosphorylation efficiency at the same concentration is 65% of that normally expected from NAD-linked substrates. The over-all efficiency of mitochondria oxidizing NAD-linked substrates in the presence of 0.48 mM AFB_1 is reduced by 63% compared to that of normal mitochondria. NAD-linked substrates were chosen as a model because the normal fuel of liver mitochondria is largely short chain fatty acids and their total oxidation is partially NAD-dependent. This effect would cause a sharp decrease in cellular ATP levels.

It is not possible for a carcinogenic dose of AFB_1 to achieve this concentration throughout the liver. It could however occur in a localized area. In the cells near the major sources of blood, the concentration of AFB_1 might reasonably be expected to be higher than in the extreme edges of the liver. One might expect a gradient of AFB_1 concentration with the higher concentration near the source. Under these conditions arriving at a suitable concentration to impair respiration would be a rare event; but since the occurrence of a neoplastic event is also rare, one can reasonably retain the theory. The literature indicates that the cells which are the most sensitive to AFB_1 are the parenchymal cells [137,138]. It might also be mentioned that the mitochondria isolated by the method employed in this study results in predominantly mitochondria from parenchymal cells. The work of Portman and Campbell indicates that the intracellular concentration of AFB_1

in liver slices achieved just prior to "cell death" was 0.158 mM [139]. Cell death is defined as a sharp reduction in 0_2 consumption. This is consistent with the work shown in Fig. 9 with submitochondrial particles. It is not however consistent with the work done using whole mitochondria. Preliminary experiments not reported in this dissertation indicate the involvement of a soluble cytoplasmic factor in the transport of AFB_1 across the inner mitochondrial membrane. The permeability of the nuclear membrane has been shown to be the limiting factor in the insensitivity of whole nuclei to AFB_1 inhibition of transcription *in vitro*. The data appear to indicate that this is also the case with the mitochondrion. An alternative explanation would be to implicate a metabolite of AFB_1 in the inhibition of oxygen consumption. The epoxide of the furano double bond was prepared by the author, and under the same conditions, it was found to be no more inhibitory than the parent AFB_1. Many derivatives of mitochondrial inhibitors, i.e., amytal and antimycin, have been prepared. The ones that have been the most potent inhibitors have had long hydrophobic side chains [140,141]. If the hydrophobic side chains were removed from antimycin or if their length is reduced, the inhibitory power decreases. It would appear on this basis that hydroxylated derivatives of AFB_1 would be no more inhibitory than AFB_1 itself. The metabolite that would appear to be the most promising inhibitor of electron transport would be one with a hydrophobic side chain on either carbons 1 or 2. This compound has been isolated and it is called 2-ethoxy AFB_1 [142].

Empirically examining the premise that rare pockets of highly

concentrated AFB_1 cause the primal carcinogenic event would be difficult if not impossible. Mitochondria that are isolated from animals that have been treated with AFB_1 regardless of the dose would result only in an average inhibition as do the results shown in this study. Difference spectroscopy of liver slices using a very small diameter light beam might be able to detect pockets of localized reduction of the electron transport system, but this would seem the only hope of examining this premise.

The possibility of an inhibition of electron transport alone being the primal event in carcinogenesis is remote. A more likely possibility would be concerted action of two or more effects of AFB_1. For example, the depression of mitochondrial efficiency and repair of mitochondria DNA. It has been shown that under conditions of energy deprivation, the repair of DNA proceeds poorly [143]. The fact discussed earlier that melanoma and hepatoma mitochondria have a deficiency in structural proteins coded for on the nuclear genome could be caused by this sort of concerted effect. The fact that primer activity has been shown to be effected by AFB_1 in *E. coli* is also pertinent. The mitochondrion has a bacterial-type of genome and one could reasonably expect a similar result to that found in *E. coli*. Tumor mitochondria resemble the mitochondria from a class of cytoplasmic mutants in yeast referred to as ρ mutants. The physiology of the members of this class of mutants resemble tumor and hepatoma mitochondria. These mitochondria have an inactive electron transport system [144,145]. On the other hand mutants of this class contain mitochondria which, when isolated,

share many of the properties found in normal mitochondria. They have the ability to change conformations, and have a full complement of characteristic enzymes such as malate dehydrogenase, isocitrate dehydrogenase, and ATPase [146,147]. These mutants cannot respire and are capable of growth only on fermentable substrates. Their metabolism is completely anaerobic. The DNA in these mitochondria is 96% adenine plus thymine [148]. These types of mutants illustrate the possibility that mitochondrial mutations can dominate the physiology of the cell. The consequence of this type of mutation in mammalian cells might be devastating. Recent work has indicated that mitochondria are, evolutionarily speaking, more recent additions to the older glycolytic form of metabolism [149]. Cancer may be a reversion to a more primitive type of glycolytic control already coded for on the nuclear genome. The losses in sensitivity to control mechanisms may be a cellular response to the conditions of energy deprivation.

The work with dietary protein deprivation shows a 42% reduction in the degree of AFB_1 inhibition compared to *ad libitum* fed controls, with restriction of food intake accounting for only 16% of this reduction (Group II versus III). The mitochondria in the protein deprived animals (Group I) appeared to be as functional as the mitochondria from the other groups, as indicated by their respiratory control ratios. In fact, Harada [150] showed that feeding male rats a 4% casein diet for 40-50 days (compared to 25% casein) was associated with a highly significant increase in the respiratory control ratios. These results, together with results of additional preparations used to determine the

ADP:O ratio indicate that mitochondrial integrity in the protein-deprived group is as good or better than either of the 20% protein groups. It is therefore possible that increased integrity of the mitochondrial inner membrane is responsible for the decrease in inhibition. The difference in the respiratory control ratio is, however, not great enough to make this very probable.

In additional experiments in this laboratory [151], it has been shown that plasma corticosterone levels of such protein-deprived rats is significantly higher (approximately 50%) than the control animals. This hormone may be a membrane stabilizer since DeDuve *et al.* [152] have shown that hydrocortisone stabilizes lysosomal membranes. Chapman and co-workers [153] have also indicated a stabilizing function for cholesterol in their phospholipid-protein model membrane systems. A stabilization of the mitochondrial membrane during protein deficiency may account for decreased AFB_1 transport into the mitochondria. There is, however, a primary difference between the inner mitochondrial membrane and other mammalian membranes and this is the fact that it contains no cholesterol. This unusual bacterial type of membrane may not be susceptible to a mammalian type of stabilization mechanism. The fact that inhibition is not increased in mitochondria with low respiratory control ratios also argues against membrane stabilization as an important factor.

There is also the possibility that the cytoplasmic factor mentioned previously is a protein. Considerable work has been done to show that protein carriers are implicated in the transport of steroids in the

cytoplasm, and protein carriers have also been implicated in the interaction of steroids with the nucleus. It is also possible that protein deficiency decreases the amount of a protein which is responsible for facilitating the transport of AFB_1 across the mitochondrial inner membrane. A thorough study will have to be made on the transport of AFB_1 across membranes before this question can be settled assuredly.

Madhavan and Gopalan have demonstrated that dietary protein deprivation of rats decreased AFB_1-induced tumor formation [154]. These same workers have shown that dietary protein deprivation promotes an increase in the acute toxic response with repeated AFB_1 doses [155]. The relaxation of AFB_1-induced inhibition of mitochondrial oxygen consumption caused by protein deficiency in these experiments would seem to agree with the findings of these workers. The work of Platonon has shown that after two weeks of feeding an aflatoxin contaminated meal the oxidized pyridine nucleotide level is significantly reduced in the liver and sera of chickens [156]. Lillehoj *et al.* [157] have suggested that these data of Platonon [156] indicate that there is a transient aflatoxin-induced disturbance of the oxidation-reduction mechanism. The experiments detailed here along with the results of others suggest that the mitochondrion may be involved in the carcinogenic process.

SUMMARY

1. The critical micelle concentration of aflatoxin B_1 was determined to be 0.48 mM in the oxygen electrode solution of Estabrook and 0.53 mM in the oxygen electrode solution of Gregg.
2. Aflatoxin B_1 was found to inhibit electron transport in rat liver mitochondria by 43% at a concentration of 0.48 mM. No increase in inhibition was observed above the critical micelle concentration.
3. Aflatoxin B_1 was found to inhibit the oxidation of NAD-dependent substrates as well as succinate.
4. The use of N, N, N', N', tetramethyl-p-phenylenediamine indicates that the site of inhibition was the second crossover point. No inhibition was observed at the first or third crossover points. The inhibition was not removed by treatment with 32 μM DNP.
5. The ADP:O ratio was depressed by 0.48 mM aflatoxin B_1 to 63% of that found in an untreated mitochondria.
6. Aflatoxin B_1 at a concentration of 0.53 mM was found to inhibit the oxidation of β-hydroxybutyrate by Gregg particles by 63%. The increase in inhibition using Gregg particles as compared to whole mitochondria is attributed to the impermeability of the inner mitochondrial membrane to aflatoxin B_1.
7. Aflatoxin B_1 was found to inhibit electron transport in Gregg particles at the second crossover point. This was confirmed by the use of menadione, TMPD and spectrophotometric techniques.
8. Protein deficiency was accomplished by feeding rats a 5% protein

diet for 15 days, and the mitochondria from these animals were 42% less sensitive to aflatoxin B_1 than mitochondria from protein sufficient animals. Gregg particles made from mitochondria isolated from animals fed a 5% protein diet showed a sensitivity to aflatoxin similar to Gregg particles from protein sufficient animals indicating permeability is reduced in mitochondria from 5% protein fed animals.

9. Animals fed a thiamine deficient diet showed an increased sensitivity to 0.1 mM aflatoxin B_1. This is attributed to an increase in permeability.

10. The inhibition of electron transport by aflatoxin B_1 in Gregg particles was found to be independent of pH from 6.5 to 8.5.

11. Dinitrophenol stimulated ATPase activity in Gregg particles is reduced in the presence of reducing substrate and aflatoxin B_1 indicating the inhibition is in the electron transport system not in the phosphorylating functions ancillary to the electron transport chain.

BIBLIOGRAPHY

1. Hiscocks, E. S. (1965). In Mycotoxin in Foodstuffs (Wogan, G. N., ed.). The Massachusetts Institute of Technology Press, Cambridge, Massachusetts. p. 291.

2. Kraybill, H. F. and Shimkin, M. B. (1964). In Advances in Cancer Research (Haddon, A. and Weinhouse, S., eds.). Academic Press, New York. Vol. VIII, p. 191.

3. Tuite, J. F. and Christensen, C. M. (1957). Phytopath., 47, 265.

4. Purchase, I. F. H. (1967). Afr. Med. J., 41, 406.

5. Sargeant, K., Sheridan, A., O'Kelly, J. and Carnaghan, R. B. A. (1961). Nature, 192, 1096.

6. Asplin, F. D. and Carnaghan, R. B. A. (1961). Vet. Record, 73, 1215.

7. Lancaster, M. C., Jenkins, F. P. and Philip, J. M. (1961). Nature, 192, 1095.

8. Schonental, R. (1961). Brit. J. Cancer, 15, 812.

9. Rogers, A. E. and Newberne, P. M. (1969). Cancer Res., 29, 1965.

10. Hartley, R. D., Wesbitt, B. F. and O'Kelly, J. (1963). Nature, 198, 1056.

11. Allcroft, R., Rogers, H., Lewis, G., Nabney, J. and Best, P. E. (1966). Nature, 209, 154.

12. Carnaghan, R. B. A., Hartley, R. D., and O'Kelly, J. (1963). Nature, 200, 1101.

13. Wogan, G. N. (1966). Bact. Rev., 30, 460.

14. Butler, W. H. (1964). Brit. J. Cancer, 18, 756.

15. Sinnhuber, R. O., Wales, J. H., Engebrecht, R. H., Amend, D. F., Kray, W. D., Ayres, J. L., and Ashton, W. E. (1965). Fed. Proc., 24, 627.

16. Wogan, G. N. and Newberne, P. M. (1967). Cancer Res., 27, 2370.

17. Sporn, M. B., Dingman, C. W., Phelps, H. L., and Wogan, G. N. (1966). Science, 151, 1539.

18. Clifford, J. I., Rees, K. R., and Stevens, M. E. M. (1967). Biochem. J., 103, 258.

19. Clifford, J. I., Rees, K. R. (1967). Biochem. J., 103, 467.

20. Black, H. S. and Jirgensons, B. (1967). Plant Physiol., 42, 731.

21. Hnilica, L. S. and Billen, D. (1964). Biochim. Biophys. Acta, 91. 271.

22. Liau, M. C., Hnilica, L. S., and Hurlbert, R. B. (1965). Proc. Natl. Acad. Sci. U. S., 53, 626.

23. Frayssinet, C., Lafarge, C., Derecondo, A. M., and Lebreton, E. (1964). Compt. Rend. Acad. Sci., Paris, 259, 2143.

24. De Recondo,A. M., Frayssinet, C., Lafarge, C., and Lebreton, E. (1966). Biochim. Biophys. Acta, 119, 322.

25. Wragg, J. B., Ross, V. C., and Legator, M. S. (1967). Proc. Soc. Exptl. Biol. Med., 125, 1052.

26. Portman, R. S. and Campbell, T. C. (1970). Biochem. Biophys. Res. Comm., 41, 744.

27. Edwards, G. S. and Wogan, G. N. (1970). Biochim. Biophys. Acta, 224, 597.

28. Klingenberg, M. (1963). In Energy Linked Functions of Mitochondria (Chance, B., ed.) Academic Press, Inc., New York, p. 381.

29. Klingenberg, M. and Pfaff, E. (1966). In Regulation of Metabolic Processes in Mitochondria (Tager, J. M., Papa, S., Quagliarello, E., eds.). B. B. A. Library, Elsevier Publ. Co., Amsterdam, Vol. VII, p. 108.

30. Pfaff, E. (1967). In Roundtable Discussion on Mitochondrial Structure and Compartmentation (Quagliarello, E., Papa, S., Slater, E. C. and Tager, J. M., eds.). Adriatica Editrice, Bari., p. 165.

31. Pfaff, E., Klingenberg, M., and Heldt, H. W. (1965). Biochim. Biophys. Acta, 104, 312.

32. Pfaff, E., Klingenberg, M., Ritt, E., and Vogell, W. (1968). European J. Biochem., 5, 222.

33. Palade, G. E. (1953). J. Histochem. Cytochem., 1, 188.

34. Klingenberg, M. (1963). In Funktionelle and Morphologische Organisation der Zelle (Karlson, P. C., ed.). Springer, Berlin, p. 69.

35. Sottocasa, G. L., Kuylenstierna, B., Ernster, L., and Bergstrand, A. (1967). Meth. Enzym., 10, 448.

36. Schwaitman, C. and Greenawalt, J. W. (1968). J. Cell Biol., 38. 158.

37. Schwaitman, C., Erwin, V. G., and Greenawalt, J. W. (1967). J. Cell Biol., 32, 719.

38. Brdiczka, D., Pette, D., Brunner, G., and Miller, F. (1968). European J. Biochem., 5, 294.

39. Norum, K., Farstad, M., and Bremer, J. (1966). Biochem. Biophys. Res. Commun., 24, 797.

40. Fernandez-Moran, H. (1962). Circulation, 26, 1039.

41. Fernandez-Moran, H., Oda, T., Blair, P. J., and Green, D. E. (1964). J. Cell Biol., 22, 63.

42. Lee, C. P. and Ernster, L. (1966). In Regulation of Metabolic Processes in Mitochondria (Tager, J. M., Papa, S., Quagliarello, E., eds.). B. B. A. Library, Elsevier Publ. Co., Amsterdam, Vol. VII, p. 218.

43. Chance, B. (1961). J. Biol. Chem., 236, 1569.

44. Gregg, C. T. and Lehninger, A. L. (1963). Biochim. Biophys. Acta, 78, 27.

45. Malviya, A. N., Nicholls, P., and Elliott, W.B. (1968). Biochim. Biophys. Acta, 153, 920.

46. Low, H. and Vallin, I. (1963). Biochim. Biophys. Acta, 69, 361.

47. Klingenberg, M. and von Jagon, G. (1970). In Electron Transport and Energy Conservation (Tager, J. M., Papa, S., Quagliarello, E., and Slater, E. C., eds.). Adriatica Editrice, Bari., p. 135.

48. Klingenberg, M. (1968). In Biochemic des Sauerstoffs (Hess, B. and Staundinger, H., eds.). Springer, Berlin, p. 131.

49. Tyler, D. D. (1969). In Biochemistry of Mitochondria (Slater, E. C., ed.). Academic Press, New York, p. 131.

50. Palmieri, F. and Klingenberg, M. (1967). European J. Biochem., 1, 439.

51. Racker, E. (1970). In Essays in Biochemistry (Campbell, P. N. and Dickens, F., eds.). Academic Press, New York, p. 1.

52. Lenaz, G. and McLennan, D. H. (1966). J. Biol. Chem., 241, 5260.

53. Lee, C. P. and Carlson, K. (1968). Fed. Proc., 27, 828.

54. Muscatello, V. and Catafoli, E. (1964). J. Cell Biol., 40, 602.

55. Estabrook, R. W. (1962). Biochim. Biophys. Acta, 60, 236.

56. Chance, B. and Williams, G. R. (1956). J. Biol. Chem., 221, 477.

57. Estabrook, R. W. (1957). J. Biol. Chem., 227, 1093.

58. Hatefi, Y. and Jurtshuk, P. and Haavik, A. G. (1961). Biochim. Biophys. Acta, 52, 119.

59. Nijs, P. (1967). Biochim. Biophys. Acta, 143, 454.

60. Teeter, M. E., Baginsky, M. L., and Hatefi, Y. (1969). Biochim. Biophys. Acta, 172, 331.

61. Burgos, J. and Redfearn, E. R. (1965). Biochim. Biophys. Acta, 100, 475.

62. Fukami, J. and Tomizana, C. (1956). Bochu-Kagaku, 21, 129.

63. Lindahl, P. E. and Öberg, I. C. E. (1961). Exp. Cell Res., 23, 238.

64. Öberg, K. E. (1961). Exp. Cell Res., 24, 163.

65. Weiner, R. F. and Frisell, W. R. (1966). Abst. Pap. 152nd meeting, Amer. Chem. Soc., p. c123.

66. Kim, S-H and Rich, A. (1968). Proc. Nat. Acad. Sci. U. S., 60, 402.

67. Ernster, L., Jalling, O., Low, H., and Lindberg, O. (1955). Exp. Cell Res., 3, 124.

68. Ernster, L., Dallner, G., and Azzone, G. F. (1963). J. Biol. Chem., 238, 1124.

69. Horgan, D. J. and Singer, T. P. (1967). Biochem. J., 104, 50.

70. Chance, B., Ernster, L., Garland, P. B., Lee, C. P., Light, P. A., Ohnishi, T., Ragan, C. I., and Wong, D. (1967). Proc. Nat. Acad. Sci. U. S., 57, 1498.

71. Greenbaum, A. L., Clark, J. B., and McLean, P. (1963). Biochem. J., 96, 507.

72. Jurtshuk, P., Sckuzu, I., and Green, D. E. (1963). J. Biol. Chem., 238, 3595.

73. Moore, J. H. and Truelove, B. (1970). Science, 168, 1102.

74. Clifford, J. I. and Rees, K. R. (1967). Biochem. J., 102, 65.

75. Svoboda, D., Gady, H. J., and Higginson, J. (1966). Path., 49, 1023.

76. Svoboda, D., Racela, A., and Higginson, J. (1967). Pharmacol., 16, 651-657.

77. Brown, J. M. M. and Abrams, L. (1965). J. Vet. Res., 32, 119.

78. Brown, J. M. M. (1965). S. African Med. J., 39, 778.

79. Warburg, O. (1923). Biochem. Z., 142, 317.

80. Warburg, O. (1956). Science, 123, 309.

81. Warburg, O. (1956). Science, 124, 269.

82. Sharma, R. M., Sharma, C., Donnelly, A. J., Morris, H. P., and Weinhouse, S. (1963). Can. Res., 25, 906.

83. Adelman, R. C., Morris, H. P., and Weinhouse, S. (1967). Can. Res. 27, 2408.

84. Farina, F. A., Adelman, R. C., Lo, C. H., Morris, H. P., and Weinhouse, S. (1968). Can. Res., 28, 1897.

85. Nowell, R. C., Morris, H. P., and Potter, V. R. (1967). Can. Res., 27, 1561.

86. Morris, H. P. (1965). Advan. Can. Res., 9, 227.

87. Greenstein, J. P. (1954). In Biochemistry of Cancer, 2nd ed., Academic Press, New York.

88. Ballard, F. J. and Oliver, I. T. (1964). Biochem. J., 90, 261.

89. Elwood, J. C., Lin, Y. C., Cristofalo, V. J., Weinhouse, S., and Morris, H. P. (1963). Can. Res., 23, 906.

90. DiPietro, D. L., Sharma, C., and Weinhouse, S. (1962). Biochemistry, 1, 455.

91. Kaplan, N. O. and Fondy, T. P. (1965). Ann. N. Y. Acad. Sci., 119, 888.

92. Penhoet, E., Rajkumar, T., and Rutler, W. J. (1966). Natl. Acad. Sci. U. S., 56, 1275.

93. Nordman, Y. and Schapira, F. (1967). Europ. J. Can., 3, 247.

94. Bailey, E. and Walker, P. E. (1969). Biochem. J., 111, 359.

95. Tanaka, T., Harano, Y., Seu, F., and Morimura, H. (1967). J. Biochem. (Tokyo) 62, 71.

96. Lo, C. H., Christofalo, V. J., Morris, H. P., and Weinhouse, S. (1968). Can. Res., 28, 1.

97. Bloch-Frankenthal, L., Langan, J., Morris, H. P., and Weinhouse, S. (1965). Can. Res., 25, 732.

98. Boxer, G. E. and Devlin, T. M. (1961). Science, 134, 1495.

99. Ohe, K., Morris, H. P., and Weinhouse, S. (1967). Can. Res., 27, 1360.

100. Wenner, C. E. and Weinhouse, S. (1953). Can. Res., 13, 21.

101. Hunter, F. E., *et al.* (1959). J. Biol. Chem., 234, 693.

102. Lehninger, A. L. (1951). J. Biol. Chem., 190, 345.

103. Hunter, F. E. and Ford, L. (1955). J. Biol. Chem., 216, 357.

104. MacLennan, D. H. and Tzagolotti, A. (1966). J. Biol. Chem., 241, 1933.

105. Ernster, L. (1956). Expt. Cell Res., 10, 704.

106. Chance, B. and Hess, B. (1959). Science, 129, 70.

107. Emmelot, P. *et al.* (1959). Brit. J. Can., 13, 348.

108. Schneider, W. C. and Hogeboom, G. H. (1950). J. Natl. Can. Inst., 10, 969.

109. Oberling, C. and Bernard, W. (1961). In The Cell (Brachet, J. and Mirskey, A. E., eds.). Academic Press, New York, Vol. V, p. 405.

110. Novikoff, A. B. (1960). In Cell Physiology of Neoplasia. University of Texas Press, Austin, p. 219.

111. Brikmayer, G. D. and Balda, Z. (1971). Fed. Eur. Biochem. Soc. Lett., 15, 156.

112. Chang, L. O., Schwaitman, C. A., and Morris, H. P. (1971). Can. Res., 31, 108.

113. Gardner, R. S. and Tompkins, G. M. (1969). J. Biol. Chem., 244, 4761.

114. Jensen, E. V., Numata, M., Brecher, P. T., DeSombre, E. R. (1971). In Biochemistry Society Symposium No. 32 (Smellie, R. M. S., ed.). Academic Press, New York, p. 133.

115. Jensen, E. V., Suzuki, T., Kanahima, T., Stumpf, W. E., Jungblut, P. W., and DeSombre, E. R. (1968). Proc. Nat. Acad. Sci. U. S., 59, 632.

116. Johnson, D. and Lardy, H. (1967). In Methods in Enzymology (Estabrook, R. W. and Pullman, M. E., eds.). Academic Press, New York, Vol. X, p. 94.

117. Estabrook, R. W. (1967). In Methods in Enzymology (Estabrook, R. W. and Pullman, M. E., eds.). Academic Press, New York, Vol. X, p. 41.

118. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951). J. Biol. Chem., 193, 265.

119. Anderson, B. M. and Anderson, C. D. (1970). Biochim. Biophys. Acta, 205, 161.

120. Lee, C. P., Sottocasa, G. L. and Ernster, L. (1967). In Methods in Enzymology (Estabrook, R. W. and Pullman, M. E., eds.). Academic Press, New York, Vol. X, p. 33.

121. Lee, C. P., Nordenbrand, K., and Ernster, L. (1965). In Proc. Symp. Oxidases Related Redox. Systems. Wiley, New York, p. 960.

122. Sanadi, D. R. and Jacobs, E. E. (1967). In Methods in Enzymology (Estabrook, R. W. and Pullman, M. E., eds.). Academic Press, New York, Vol. X, p. 38.

123. Fiske, C. H. and Subbarow, Y. (1925). J. Biol. Chem., 66, 375.

124. Galeotti, T., Angelo, A., and Chance, B. (1970). Biochim. Biophys. Acta, 197, 11.

125. Mgbodile, M. U. K. and Campbell, T. C. (1972). J. Nutr., 102, 53.

126. Borst, P. (1962). Biochim. Biophys. Acta, 57, 256.

127. Quagliarello, E. and Papa, S. (1964). In Atti del Seminariodi Studi Biologici, Cressati, Bari. Vol. I, p. 351.

128. Papa, S., Palmieri, F. and Quagliarello, E. (1966). In Regulation of Metabolic Processes in Mitochondria (Tager, J. M., Papa, S., Quagliarello, E., and Slater, E. C. eds.). Elsevier Publishing Co., New York, Vol. VII, p. 153.

129. Devlin, T. M. and Bedell, B. M. (1960). J. Biol. Chem., 235, 2134.

130. Hemker, H. C. (1964). Biochim. Biophys. Acta, 81, 9.

131. Conover, T. E. and Ernster, L. (1962). Biochim. Biophys. Acta, 58, 189.

132. Mustata, M. G. and King, T. E. (1967). Arch. Biochem. Biophys., 122, 501.

133. Hatefi, Y., Haavik, A. G., and Griffiths, D. E. (1962). J. Biol. Chem., 237, 1681.

134. Hatefi, Y., Haavik, A. G., and Jurtshuk, P. (1961). Biochim. Biophys. Acta, 52, 106.

135. Frei, J. and Ryser, H. (1956). Experientia, 12, 105.

136. Jacobs, E. E. and Sanadi, D. R. (1960). J. Biol. Chem., 235, 531.

137. Terao, K. and Miyaki, K. (1968). Z Krebstorsch, 71, 199.

138. Rogers, A. E. and Newberne, P. M. (1967). Can. Res., 27, 855.

139. Portman, R. S., Plowman, K. M., and Campbell, T. C. (1970). Biochim. Biophys. Acta, 208, 487.

140. Dickie, J. P., Loomans, M. E., Farley, T. M., and Strong, F. M. (1965). J. Med. Chem., 87, 3501.

141. Conger, M. L., Labbe, R. F., and Mackler, B. (1962). Arch. Biochem. Biophys., 96, 583.

142. Andrellos, P. J., Beckwith, A. C., and Eppley, R. M. (1967). J. Assoc. Office. Anal. Chem., 50, 346.

143. Moss, A. J., Dalrymple, G. V., Sanders, J. L., Wilkinson, K. P., and Nash, J. C. (1971). Biophys. J., 11, 158.

144. Sherman, F. and Slonimski, P. P. (1964). Biochim. Biophys. Acta, 90, 1.

145. Mackler, B., Douglas, H. C., Will, S., Hawthorne, D. C., and Mahler, H. R. (1964). Biochemistry, 4, 2016.

146. Kovac, L. and Weissova, K. (1967). Biochim. Biophys. Acta, 153, 55.

147. Schatz, G. (1968). J. Biol. Chem., 243, 2192.

148. Bernard, G., Carnevali, F., Nicolaieff, A., Piperno, G., and Tecce, G. (1968). J. Mol. Biol., 37, 493.

149. Sagan, L. (1967). Theoret. Biol., 14, 225.

150. Harada, N. (1967). J. Nutr., 93, 263.

151. Mgbodile, M. U., Ph.D. Thesis, Virginia Polytechnic Institute and State University, Blacksburg, Virginia, 1972.

152. DeDuve, C., Wattiauz, R., and Wibo, M. (1962). Biochem. Pharmacol., 9, 97.

153. Chapman, D. and Wallach, D. F. H. (1968). In Biological Membranes, Physical Fact and Function (Chapman, D., ed.). Academic Press, New York, p. 125.

154. Madhavan, T. U. and Gopalan, C. (1968). Arch. Path., 85, 133.

155. Madhavan, T. U. and Gopalan, C. (1965). Arch. Path., 80, 123.

156. Platonon, N. (1965). Can. J. Comp. Med. Vet. Sci., 29, 23.

157. Lillehoj, E. B., Ciegler, A., and Detroy, R. W. (1970). In Essays in Toxicology (Blood, F. R., ed.). Academic Press, New York, Vol. II, p. 1.

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EFFECT OF AFLATOXIN B_1 ON MITOCHONDRIAL FUNCTION

by

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(ABSTRACT)

The mycotoxin, aflatoxin B_1, elaborated by the fungus *Aspergillus flavus* is a coumarin-type compound. This compound has previously been reported in the literature to have an effect on rat liver mitochondria, although the reports are inconsistent. The effect of this compound on oxygen consumption by rat liver mitochondria was examined potentiometrically. All experiments were carried out below the critical micelle concentration of aflatoxin B_1 which was determined in the oxygen electrode solution of Estabrook to be 0.48 mM. Mitochondria were isolated from 80-150 g male Sprague-Dawley derived rats. Aflatoxin B_1 at a concentration of 0.48 mM was found to inhibit oxygen consumption by 43% in the presence of ADP and by 63% in the presence of DNP. The inhibition brought about by 0.48 mM aflatoxin B_1 occurs when either succinate or β-hydroxybutyrate is used as a substrate. When electrons were fed into the electron transport chain at cytochrome c_1 (or c) using TMPD and ascorbate no inhibition was observed. TMPD, however, released the inhibition brought about by 0.48 mM aflatoxin B_1 in the presence of succinate or β-hydroxybutyrate as substrates. No relief of the inhibition was obtained with menadione. The site of inhibition appears to be between cytochrome b and cytochrome c_1 (or c). The ADP:O ratio was also depressed by 35% in the presence of 0.48 mM aflatoxin B_1 when NAD-dependent substrates were employed.

Mitochondria from protein deficient animals were found to be 42% less sensitive to inhibition by aflatoxin B_1 than mitochondria from protein sufficient animals. The ADP:O ratio with NAD-dependent substrates was found to be 37% less sensitive in mitochondria from protein deficient animals than in protein sufficient animals.

Gregg particles were prepared from mitochondria isolated from protein deficient as well as protein sufficient animals. Both types of submitochondrial particles were found to be more sensitive to aflatoxin B_1 than whole mitochondria. It was concluded that the inhibition was limited in whole mitochondria by the inner mitochondrial membrane; and in protein deficient mitochondria there is an alteration in the inner mitochondrial membrane which makes aflatoxin B_1 a less potent inhibitor of electron flow.

The fact that the inhibition occurs in the presence of DNP as well as ADP indicates that the inhibition is in the electron transport chain and not in the phosphorylating functions ancillary to the chain. DNP-stimulated ATPase activity was only slightly affected by aflatoxin B_1 in the absence of reducing substrates. DNP-stimulated ATPase activity was however markedly reduced in the presence of reducing substrate and aflatoxin B_1. This supports the contention that aflatoxin B_1 effects electron transport in the chain and not in the phosphorylating functions ancillary to the chain though some effect is seen there also.