Determining Safe Levels of Mycotoxins

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ABSTRACT

The establishing of safe levels of mycotoxins to date has been a legal rather than scientific exercise. This has resulted in levels which have varied in response to economic and political pressures. The data base for rationally determining safe levels is very small. This has resulted in subjective evaluations in levels which have varied in response to economic and political factors, and in assumed safe levels which vary from field experiences. Using physiological parameters other than growth as criteria of safety, known deleterious interactions of mycotoxins with other factors, and statistical corrections for inadequate numbers of animals tested, permit better agreement between safe levels determined from laboratory data and from field data. However, the number of animals required makes impractical the laboratory determination of truly safe levels. Well-conceived and executed epidemiological studies coupled with laboratory studies designed to elaborate underlying principles appear to be the best approach to determining safe levels of mycotoxins. Until safe levels are based on sound animal experimentation, the prudent person would assume there is no truly safe level and that increasing levels of mycotoxins carry increasing risk.

If it has been asked once, it has been asked 10,000 times, “What are safe levels of mycotoxins?” It is a very simple, obvious and intensely practical question which deserves a simple, straightforward answer. Unfortunately, this question has been poorly investigated. The main reasons for this inactivity have been that we have taken refuge in regulatory guidelines promulgated to satisfy legal requirements rather than scientific requirements and that, in consequence, we have abdicated our responsibility for the orderly and logical development of a new area of science.

It is not always appreciated that regulatory agencies sometimes are required by law to adopt positions that cannot be justified scientifically and that these positions can change with time for many reasons. Aflatoxin was discovered in 1961 and its potential as a carcinogen was reported in 1962. In response, the Food and Drug Administration (USA) promulgated in 1965 a guideline on aflatoxin of 30 ppb. This value was the least that could be reliably detected by the available analytical methods and hence qualified as the zero amount mandated for carcinogenic food additives by the famous Delaney Amendment. As analytical methodology improved, the zero amount became 20 ppb in 1969, where it remained for several years. A consequence of the strict legal requirement and the inevitable improvement of detection methods was the placing of FDA in the untenable position of having to condemn the entire USA corn crop, for example, if it should possess more than 20 ppb of aflatoxin. Luckily for everyone, Lillehoj et al. (10) discovered, as the result of some keen detective work, that aflatoxin could be produced in corn before harvesting. Aflatoxin then became a naturally occurring carcinogen not formed necessarily during faulty storage and could be viewed essentially as an act of God rather than a food additive under the control of man. Thus, FDA could logically exempt aflatoxin from regulations pertaining to food additives.

This flexibility was sorely needed in 1977 when aflatoxin commonly occurred at levels greater than 20 ppb in corn in the southeastern USA. The orderly marketing of corn essentially stopped in the affected states and unregulated black markets commenced because crops had to be harvested, animals had to be fed and obligations had to be repaid regardless of legalities. The high incidence of aflatoxin in corn presented for sale in one affected state, North Carolina, during 1977 through 1980 is shown in Table 1. Acting in a judicious manner, the FDA, on application by an affected state, raised the guideline for aflatoxin to 100 ppb for intrastate use as long as the contaminated corn or its products did not enter human food and animal health was not affected. The guidelines reverted to 20 ppb the next crop year, until 1980, which was another crisis year for aflatoxin in southeastern corn. FDA raised the guideline to 200 ppb for intrastate use and to 100 ppb for interstate use if the receiving state certified awareness and willingness to abide by the remaining restrictions. Material containing up to 400 ppb could now be blended with uncontaminated corn to meet the new restrictions. [See Hamilton (6) for more details.] More recently, the FDA responded to a crisis in Arizona cottonseed by allowing cottonseed meal containing up to 300 ppb aflatoxin to be marketed as long as it was fed to mature, non-lactating animals. These more realistic
TABLE 1. Incidence of aflatoxin contamination in corn offered for sale in eastern North Carolina.

<table>
<thead>
<tr>
<th>Range (ppb)</th>
<th>Crop year</th>
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<tbody>
<tr>
<td>Negative</td>
<td>37.7*</td>
</tr>
<tr>
<td>1-19</td>
<td>14.5</td>
</tr>
<tr>
<td>20-99</td>
<td>33.3</td>
</tr>
<tr>
<td>100 +</td>
<td>14.4</td>
</tr>
</tbody>
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*Tabular values are percentage of samples tested within given range. Samples were taken on a random survey basis from farmer trucks at point of sale. The study was conducted jointly in 1977 and 1978 by North Carolina State University, North Carolina Department of Agriculture, and U.S. Department of Agriculture. The 1979 and 1980 data were collected by the North Carolina Department of Agriculture.

guidelines successfully removed a heavy, if not impossible, financial burden from corn and cottonseed producers and purveyors of their products. Unfortunately, much of the burden appears to have been shifted to animal producers and other consumers.

Thus, safe levels of aflatoxin have not been immutable and the setting of safe levels has had several components: (a) legal requirements, (b) sensitivity of the analytical methods, (c) presumed safety to animals and humans, and (d) hovering over all these considerations has been that of economics or what level can be afforded. It should be noted that in all the foregoing considerations the word safe has never been defined. Further, the database regarding safety of aflatoxin and other mycotoxins is practically nonexistent. The few existing data were usually obtained ancillary to establishing an effect of a toxin. This void of information has forced regulatory personnel responsible for establishing guidelines to make subjective judgments about the value of different studies (13). In any scientific attempt to establish a safe level of a mycotoxin, it seems essential to define safety and criteria to be used in establishing it. For example, 2% mortality is a goal in some broiler chicken operations in the USA, whereas 8% mortality is acceptable in other operations. Since mortality caused by mycotoxins is rare compared to growth depression, a case can be made for using growth depression as a primary criterion. In brief, there are many ways to attack this problem of establishing a safe level.

In a thoughtful synthesis, van Rensberg (15) combined the data from several independent studies which attempted to determine the amount of aflatoxin consumed by populations with differing rates of cancer. A straight line fitted the data very well; indeed the correlation coefficient between aflatoxin consumption and liver cancer was an impressive 0.96. If one assumes that a linear relationship holds over all concentrations (an assumption believed false by many), extrapolation to intersection with the x-axis yields the safe level or minimal effective dose (MED) of aflatoxin. This would seem to be a beautiful approach until it is realized that each of the independent studies was flawed on methodological grounds and on the fact that the aflatoxin level measured was not necessarily the level which the population consumed twenty or thirty years earlier which is the latent period for the development of primary cancer of the liver. Further, the FDA conducted a retrospective study (Bureau of Foods, FDA, internal document, 1978. Assessment of estimated risk resulting from aflatoxins in consumer peanut products and other food commodities; unpublished) in the United States based on the assumption that rural Southerners consumed more corn and peanuts than urban Southerners who consumed more than urban Northerners. Corn and peanuts have the highest concentration of aflatoxin of any substances in the American diet. From the cancer registries in these geographic areas, FDA obtained the rates of liver cancer. Surprisingly, the rural Southerner had the lowest rate of liver cancer and the urban Northerner had the highest rate. If one insists on a relationship between aflatoxin and liver cancer, one then is led to the conclusion that aflatoxin had a protective effect against liver cancer. Helpful as the foregoing studies may be, it is obvious that they cannot stand on their own and must be supplemented with more rigorous data.

Animals, unlike humans, make nice experimental subjects and have been used in many attempts to define safe levels of mycotoxins. In a well-explored experimental model, the growth of young broiler chickens is inhibited by 2.5 ppm dietary aflatoxin but not by 1.25 ppm (72). Such data have been interpreted as meaning that aflatoxin below 2.5 ppm is safe for chickens. Such interpreters ignore the fact that the experimental model was designed to show an effect of aflatoxin on parameters such as susceptibility to bruising (14), not to show a safe level. They also ignore completely data in the same publication that showed a level below 0.625 ppm (the lowest level tried) can affect susceptibility. Similar data showing an MED below 0.625 ppm on pancreatic trypsin, serum lipids, serum iron, bile salts, plasma prothrombin and the primary immune response are ignored also.

These oversights raise the question of what is the proper criterion of safety. Is it cancer which doesn't occur in this model, is it growth rate, is it susceptibility to bruising or some other variable? Apparently most people prefer growth rate, but this preference needs examination. Obviously, there are several physiological parameters in broiler chickens more sensitive to aflatoxin than is growth rate, but some people question the importance to the animal of alterations in such parameters if they do not correlate with something like growth which is "self-evidently" important. In answer, animal physiologists say that any change from normal will be a disadvantage under some circumstances. Selvy (77), in his discourse on the General Adaptation Syndrome, expounded on what he called countercurrent stress in which stressors below minimally effective levels combine to cause an effect. In more common microbiological terms, apparently ineffective factors interact synergistically.

Let us examine some mycotoxical data from the viewpoint of this fundamental physiological principle and
see its unifying role in determining and understanding safe levels of mycotoxins. The percentage phagocytosis by heterophils in broiler chickens showed an MED between 0 and 0.625 ppm, the lowest level tried (2). Similar data were obtained for serum complement and the production of antibodies. Obviously, the immune system is impaired during aflatoxicosis. Those obsessed with growth as the criterion of safety would ignore such data. However, when birds consuming aflatoxin were infected with Salmonella worthington, the minimal growth inhibitory level decreased from 2.5 ppm to <0.625 ppm (7). An identical effect on growth was obtained when aflatoxin was added to a diet deficient in riboflavin or vitamin D (7). It seems fair to conclude that physiological abnormalities other than decreased growth rate are valid criteria for defining safe levels of aflatoxin. Stated more picturesquely, apparently healthy animals can be poised on a precipice over which they will plunge as the result of an otherwise innocuous nudge.

The preceding off-range data can be utilized to estimate an MED more precisely than <0.625 ppm, if an assumption is made about the shape of the dose response curve. An assumption of a straight-line relationship which agrees with the visual impression gives rise to a simple equation: \[ Ay = \frac{Ax}{AY} \]
where \( b = \text{slope of assumed line} \) and \( \Delta y = \text{response caused by} \) \( \Delta x = \text{change in toxin concentration} \). For calculation of MED from off-range data set \( \Delta y = \text{least significant difference as determined by analysis of variance.} \) With this simple-minded approach, six variables whose MEDs in the experiments were <625 ppb had calculated MEDs ranging from about 200 to 350 ppb. Thus, using literature data from experiments designed to show effects rather than safe levels, we can define the safe level of aflatoxin in broiler chickens as being approximately equal to the current FDA guidelines. If the same ratio between growth inhibitory dose and physiologically effective dose (i.e., about 10:1) applies when single interactions occur, then an MED of about 20 to 35 ppb, approximately equal to the old FDA guidelines, can be calculated.

Next, let us consider some experiments designed to define a safe level of aflatoxin. In this series of experiments, egg-type chickens, which are more resistant to aflatoxin than meat-type or broiler chickens, were used because of cost and ease of acquisition. The influences of number of replicates per treatment, of dosage increments, and of method of statistical analysis on the apparent MED of aflatoxin on four dependent variables were investigated (4). Doubling the number of replicates per treatment from four to eight roughly halved the apparent MED using a customary analysis of variance in which the least significant difference between treatments is calculated if the F-ratio is significant (\( P<0.05 \)). Decreasing by a factor of eight the usual two-fold dosage increments almost halved the apparent MED again. However, the foregoing statistical analyses compare each treatment mean with an experimentally determined control mean which has its own experimental error. Consequently, the full information in the data cannot be extracted and the apparent MED will generally be inaccurate.

This limitation can be overcome by a mathematical modeling approach which provides a much more accurate estimate of MED while providing information about the shape of the dose-response curve. First, the plotted data are examined visually for their approximation to various types of dose-response curves. Then an equation for the dose-response curve is obtained by least-squares methods. The fit of the obtained equation to the data is then tested by an analysis of variance. From the equation thus developed, a predicted response curve is calculated with confidence limits. Even the control value is predicted which removes a large source of error in estimating MED. Finally, the MED for a variable can be calculated iteratively or somewhat less accurately by graphical estimation (see Dixon et al. (4) for details).

This approach revealed differing shapes of dose-response curves for the differing variables (Fig. 1). Responses of body weight and relative weight of the bursa of Fabricius were described by quadratic polynomials. The response of the liver was fit better by a plateau-linear model with a definite threshold. The response of the pancreas was best fit by a linear-plateau model which has two intersecting sloping lines. These results suggested more than one mechanism of action for aflatoxin, that the MED depends on the variable studied, and that assumptions about dose-response relationships of aflatoxin could be better founded.

Using this approach, the apparent MED of aflatoxin on the pancreas in egg-type chickens was reduced by a factor of nine, from 4.0 ppm in the customary laboratory model to 0.43 ppm. Overall, this approach decreased the apparent MED of aflatoxin on four variables by a factor of

![Figure 1. Idealized dose-response curves for different variables during aflatoxicosis in chickens.](image-url)
of four. Applying this factor of four to the earlier results with normal broiler chickens (estimated MED = 200 to 350 ppb) yields an MED of 50 to 88 ppb. It should be emphasized that this correction factor has to do with numbers and not with the biology of the birds' response, hence it is a valid correction factor. The question arises of how far this approach can be taken. There is a simple equation for calculating the number of replicates required to detect given differences in a variable (3). Assuming an experimental design identical to that of Dixon et al. (4) and assuming a typical coefficient of variation (4.3%) obtained in such experiments, about 400 replicates per treatment would be required to detect true differences of 1% in body weight. This means about 8,000 pens and 80,000 birds would be required which is impractical if not impossible under controlled laboratory conditions permitting isolation of single variables.

Does a 1% difference in body weight, feed conversion or similar variable have any practical significance? The answer is yes, as a 1% difference in body weight and feed conversion in the broiler chicken industry was calculated to cost the USA $92,000,000 annually (Table 2). It is a small wonder that these two variables are closely monitored in the poultry industry.

Is there any way the exorbitant experimental determination of a true MED can be avoided? There are theoretical ways which require assumptions about the dose-response curve, and such assumptions are demonstrably precarious (Fig. 1). Nevertheless, let us assume some continuous relationship between aflatoxin and body weight of birds with no threshold dose, and increase the number of birds to 10,000 per unit which approximates the numbers at risk in small poultry operations. Because the standard error of a treatment varies inversely with the root mean of the number of observations upon which the treatment mean is based, it follows that the MED would be lowered by a factor of 100. Applying this factor to the broiler chicken experiments gives an MED of <1 ppb. Applying it to the body weight of egg-type chickens yields of MED of 10 ppb. Unfortunately, such numbers of birds can be considered infinitely large, i.e., they are populations not samples upon which statistics are based. This means any difference between populations is significant, and it means we cannot predict an MED for a population from experiments with small numbers. Consequently, experiments with large numbers must be run or we have to rely on field observations for a practical MED.

**TABLE 2. Cost of one percent change in growth rate and feed conversion of broiler chickens.**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Cost/Bird ($)</th>
<th>Cost for USA ($)/yr</th>
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<tbody>
<tr>
<td>Growth rate</td>
<td>0.017</td>
<td>65,000,000</td>
</tr>
<tr>
<td>Feed conversion</td>
<td>0.007</td>
<td>27,000,000</td>
</tr>
</tbody>
</table>

*Cost of feed = $175/ton (1982 estimate).*  
*Number of USA broilers = 3.8 × 10^9 (USDA estimate, 1979).*

If matters were not difficult enough, there are other complications to consider. Liver lipid increases during aflatoxicosis (12) and can be a criterion for safety. However, ochratoxin prevents the accumulation of lipid during aflatoxicosis (W. E. Huff, personal communication). If an MED for aflatoxin based on liver lipid were being determined, the unknown presence of ochratoxin would give falsely high values. Lillehoj and Ciegler (9) reported an example of a deleterious interaction in which doses of penicillic acid and citrinin were innocuous when administered alone but gave 100% lethality in combination. Such interactions occur commonly and over a dozen factors interact with aflatoxin. A difference of up to 250% in oral LD_{50} of aflatoxin in chickens, depending on the strain of chicken, has been reported (12). The symptoms of aflatoxicosis also depend on the strain. Duplicating in a controlled manner the many variables, some of which may be unknown, that interact in the field to produce numerically small but economically large effects appears so formidable that epidemiological studies might yield a more accurate and less expensive MED.

The available field data on a practical MED for aflatoxin confirms the foregoing considerations. An integrated broiler chicken operation with two mills receiving feed ingredients from the same source, using the same diet formula and the same bird management had a 1% difference in feed conversion and a 2% difference in body weight depending on the mill supplying the feed (5). At today's prices (Table 2), the difference in cost of production was 4¢ per bird or $400,000 for the 10,000,000 birds affected. The highest level of aflatoxin found in the problem feed was 30 ppb and only 30% of the feed samples were contaminated, whereas in the good feed the highest aflatoxin level was 6 ppb and only 2% of the samples were contaminated. The presence or absence of other factors was not known, but clean-up and the use of a mold inhibitor solved the problem with productivity while reducing the level and frequency of aflatoxin contamination in the problem mill to those in the normal mill.

A survey of five independent broiler chicken operations whose growers were classified as good, mediocre or poor based on an objective index of their productivity the prior year yielded similar results (8). In one grow-out cycle (hatching to market), the good growers produced more birds with better body weight, feed conversion and livability and with fewer condemnations at the processing plant than the mediocre and poor growers. In consequence, the good growers received 12.0¢ per chicken, the mediocre growers received 11.5¢ per chicken, and the poor growers received 10.9¢ per chicken. There was a negative correlation between each productivity factor and the level and frequency of aflatoxin in the feed consumed. The good growers had a contamination frequency of 18% and the mean aflatoxin concentration in the positive samples was 6.1 ppb. The comparable values for the poor growers were 31% and 14.0 ppb. None of the 394 samples in the survey contained more than 200 ppb aflatoxin, and only three samples contained more than 100 ppb.
The survey design ensured that growers received feed with the same level and frequency of contamination. These epidemiological studies suggest that the MED for aflatoxin in broiler chickens is below 10 ppb under field conditions where aflatoxin can interact deleteriously with many other factors. The value agrees reasonably well with values calculated and projected from laboratory studies and theoretical considerations. This satisfying coincidence should not make us overlook the fact that it appears virtually impossible at present to define an MED under laboratory conditions that will be accurate under field conditions. These studies highlight yet another difficulty of defining an MED when only a small percentage of the feed samples are contaminated and the levels of contamination are variable and unknown. Despite the difficulties, direct attempts to define safe levels of mycotoxins should continue if only for the new principles and approaches that might be discovered.

Our current knowledge about safe levels of mycotoxins can be summarized (Fig. 2) by plotting risk incurred against concentration of mycotoxins consumed. This idealized illustration assumes a proportionality between risk and mycotoxins. There is a zero intercept without a threshold. At our present state of knowledge there do not appear to be truly safe levels of mycotoxins. The prudent person will probably assume that any level carries a risk. The higher the concentration and frequency of mycotoxin exposure, the higher the risk. The presence of accessory interactants increase the risk. It should be noticed that the y-axis is labeled risk rather than response. The importance of interacting factors to the manifestations of mycotoxins means that a given amount of toxin will not ensure a given response under field conditions. This uncertainty is conveyed by the upper line in Figure 2. These considerations suggest there is no magic number above which it is safe and below which it is un-safe. Further, our attentions should be directed to reducing the risk. Regulatory actions which "liberalize" guidelines for the consuming animal do not appear justifiable on the basis of a lack of animal risk or response. Rather they are justifiable on economic grounds by permitting the use of contaminated materials otherwise destined for disposal, and by transferring or sharing the economic burden from the producer to the consumer. The use of economic criteria in setting safe or acceptable levels of mycotoxins has taken a vitally important task out of the laboratory and away from the scientist. Perhaps it was our neglect of this task which permitted its transfer. It would seem to be our duty to establish rational principles allowing enlightened control and disposition of mycotoxins in the future.

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REFERENCES