and tissue levels are not increased or clearance time delayed. In Table II, the data illustrate that the presence of zoalene did not alter the blood and tissue levels of chlortetracycline. Nor was there an effect on the comparable clearance time, although these data are not shown. As in the previous experiment, an organism, *Bacillus cereus* var. *mycoides*, known to be sensitive to chlortetracycline and not to zoalene, was used as the assay organism. Thus, the same conclusions may be drawn from the two experiments—namely, efficacy was maintained and compatibility or noninterference of agents was observed. Conversely, the presence of chlortetracycline and other commonly used antibiotics in the diets of chickens did not alter the blood and tissue levels of zoalene (*1*). Similar observations have been made with other agents, such as nystatin, N.F.-180, and hygromycin in broilers, and reserpine and nystatin in turkeys.

Another example of compatibility involves three antibacterial agents. The data in Table III illustrate the similarity of levels of chlortetracycline in blood and tissues when groups of pigs were fed rations containing chlortetracycline; chlortetracycline and sulfamethazine; or chlortetracycline, penicillin, and sulfamethazine. The data in Table IV similarly illustrate that sulfamethazine levels in swine blood and tissues were not affected by the feeding of other agents. The differences between the average values in the tables are within the expected range of biological variation. The data on the influence of the other two agents on penicillin blood and tissue levels are not shown because the levels were zero. Tests have also shown that hygromycin and nystatin do not affect the efficacy or blood and tissue levels of chlortetracycline in swine.

Thus, the author's experiences indicate that interference with the efficacy or blood levels of chlortetracycline does not occur when used in combination with previously declared safe individual agents. The agent is obviously present in its active form when the assay method is critical enough to determine its presence. Generally, agents are specifically different in their molecular structures and actions so that interference by following similar metabolic pathways within the body is unlikely, and too, the agent in numerous cases is absorbed and then eliminated as the intact or slightly modified molecule.

At the present time, compatibility of agents within a combination must be proved by two separate experiments:

The effects on absorption, excretion, and tissue residues are determined by assays of blood and tissues from animals given each specific agent alone and animals given the combination in question.

The effect on efficacy is determined by comparing the results of treating animals experimentally infected with specific diseases with the agent alone or with the combination in question.

When the results of these two tests are the same for the combination groups as for those treated with the one agent, it is concluded that they are completely compatible. To facilitate the clearance of more combinations, it seems that the induced disease experiment could be safely eliminated for those agents requiring microbiological assay procedures, and even for those agents that are determined by chemical procedures where their presence alone has previously shown them to control a condition effectively. In the case of antibiotics, if equal blood levels are found, the antibacterial activity has not been affected by the combination since blood levels are determined by a microbiological method. Thus, there is little reason to believe that other sensitive organisms, such as those that would be used in an induced disease test, would respond differently.

These statements are based on the observation that blood and tissue levels are the same when the agent is used alone as when the combination of agents is used. Under these circumstances, only blood level data should be required.

It may be argued further that when the blood levels of the combination-treated groups are the same as the blood levels of the single agent-treated group, it becomes evident that the combination of agents did not destroy the single agent while in the feed or in the gut of the animal; the agent's absorption or excretion was not changed by the presence of the other members of the combination; the single agent has not been altered in its metabolism by the combination; and the tissue residues have not been increased by the combination.

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FEED ADDITIVES

The Impact on the Analytical Chemist of Government Regulations Pertaining to Tissue Residues

E. E. KENNEDY
Analytical Research and Development Laboratories, Eli Lilly and Co., Indianapolis, Ind.

CHEMICAL SUBSTANCES added to animal feeds and fed to food-producing animals may produce residues of the substance in the animal tissue by indirect means. These residues are regarded as food additives and are regulated accordingly under Section 409 of the Federal Food, Drug, and Cosmetic Act. Pesticide chemicals may be used on raw agricultural commodities and may also cause residues in or on the plant tissue. These residues are regulated accordingly under Section 408 of the Act. It is this type of indirect additive, or residue, which will be discussed in this paper.

The problems associated with directing analytical work in measuring residues become extremely complex in view of current regulations and the interpretation of these regulations. This paper will discuss some of these complexities and will suggest possible solutions to some of the problems involved in complying with the regulations. It is hoped that through continued discussion of the many ramifications of the regulations that industry and the FDA will eventually agree on reasonable solutions to their mutual problems.

Problems Associated with "Zero" Residue

It is generally agreed that absolute zero is impossible to attain with present analytical methodology, since it would be necessary to detect one molecule of substance per some quantity of tissue. Residues must therefore be considered in terms of "relative zero." FDA regards relative zero as the level represented

A "less than" tolerance should replace the "zero" tolerance since the latter term becomes essentially meaningless when dealing with quantities such as those found in today's residue laboratory. What is a zero residue today may well be a positive residue tomorrow simply because of the progress in the field of trace analytical techniques. In establishing residue tolerances based on sensitivity of method, consideration should be given to the relative degree of confidence used to establish the sensitivity; the inherent variability in analytical methodology; and animal-to-animal and plant-to-plant background variation. The sensitivity of the method as a range rather than as a single value should be established, and reference to the analytical method should be made at the time the regulations establish a tolerance. The sensitivity range should also be included with the method.

by the sensitivity of the method. This appears to be a reasonable definition. However, just how does one define sensitivity of method? A consensus of opinion does not exist on this point. If sensitivity of method is defined as the lowest level of detection where a significant measurable difference exists between control, and control plus known amount of substance to be measured, then how statistically significant should this difference be? There needs to be some general agreement of concept established on this point, since the interpretation of data used in determining sensitivity could vary considerably among analytical chemists and microbiologists making such measurements. Therefore, relative zero may be considered to be dependent upon the sensitivity of method and the statistical probabilities used in defining sensitivity.

A second factor upon which relative zero is dependent is the inherent variability of assay method. For example, the chemical methods of determining residues usually have less variability than the microbiological methods. Suppose that antibiotic residues are to be measured by a microbiological method. Certain unknown factors can contribute to assay variation on the same sample. These tests may show a residue on several days testing which disappears on repeated testing. Or a residue may be observed in retesting, where none occurred in the original test. This situation makes it difficult to establish whether there is less than "relative zero" residue present. Since some of the measurements were above the apparent sensitivity of method, it might be concluded that a residue was present. On the other hand, if far more of the measurements were below the apparent sensitivity of method, it might be concluded that no residue was present. Unless a good estimate of variability of the method at the point of sensitivity is available, it would seem to be extremely difficult to come to either conclusion with any degree of confidence. If the true value of a tissue residue were at exactly the point of estimated sensitivity of the method, it would be expected that 50% of the measurements would be above this point and 50% below. Therefore, the sensitivity of method should be stated as a range rather than a specific value. One factor involved in estimating this range would be the variability of the method.

A third factor making relative zero, or sensitivity of method, difficult to establish is the fact that there is plant-to-plant or animal-to-animal background variation. Background here refers to the effect of the control or blank tissue upon the system of measurement used in the analytical method. Possibly background variation of tissue taken from different animals will be even greater than that of the analytical method. Therefore, background variation from control, or blank tissue, must also be a factor in determining the sensitivity range, or relative zero. All sources of variation must be included in establishing the over-all variability of the test procedure. All variations need not be given classification, but one must recognize that such variation exists.

The following scheme is given as a proposed means of obtaining a sensitivity range which will account for the variabilities discussed above:

—From replicate measurements on control tissue, and on control tissue to which has been added varying quantities of substance to be measured, determine the lowest concentration of substance where a statistically significant difference from control is obtained at the 95% confidence level.

—Control tissues from different animals, and these same tissues to which has been added the significant concentration of substance are then tested in replicate each day over a period of several days.

—From the above series of measurements, the mean difference between control, and control plus added substance, can be calculated. Also from these data, the standard deviation of the differences can be calculated as can the standard error for averages of n determinations.

—From these data, a sensitivity range of the test procedure for n determinations can be stated in terms of the standard deviation.

—In defining the upper limit of the sensitivity range, or relative zero, the risk of calling positive a sample having a true mean residue value within the sensitivity range, and the risk of failing to call positive a sample having a true mean residue value above the upper limit of the sensitivity range must be considered.

A fourth factor complicating the establishment of relative zero stems from the use of radiochemical techniques for residue measurement. If a faint trace of radioactivity is found in tissues, it could be far below the sensitivity of a practicable working method which one can submit with his petition for establishing a tolerance of relative zero. Even though the practicable chemical method establishes the fact that the residue level is below the sensitivity of the method and therefore is relative zero based on the method, the fact that a trace of residue is present as demonstrated by radiochemical techniques makes it difficult to rationalize the establishment of a zero tolerance.

A fifth factor which complicates the use of relative zero tolerance is illustrated in the following example. Suppose that a zero tolerance has been established by the FDA as a result of the data obtained by a given analytical method having a sensitivity of 1 p.p.m. With the growing field of trace analytical techniques, a method will likely be devised by either the original petitioner or the FDA laboratories that will have a greater sensitivity for the particular residue than the one originally used to establish a tolerance. Now when the tissues are tested, 50 p.p.b. of the residue are found. Should the fact that 50 p.p.b. exist be ignored, or should a new positive tolerance of less than 50 p.p.b. then be issued? As more sensitive methods are developed this could mean the amending of tolerances quite frequently from relative zero to a positive tolerance which would be below the level of the sensitivity of the original method.

From a scientific and logical point of view, a more reasonable way to establish a tolerance based on sensitivity of method is by the use of a "less than" statement with reference to a specific method having a certain sensitivity

range. The "less than" amount would refer to the upper limit of the sensitivity range. It is known that analytical chemistry has advanced not only in terms of specificity of methodology but also in terms of sensitivity. This trend may reasonably be expected to continue. It is, therefore, proposed that the "less than" tolerance be used in place of "zero" tolerance, and in this manner problems created by new methodology being developed with greater sensitivity would be eliminated.

The analytical method should be included in the regulations establishing a tolerance. If the method has been published, the regulations could refer to the published method. If the tolerance established is based on the limit of sensitivity of the method, the regulations should also state the sensitivity range.

Under pesticide chemicals regulation 120.7, provision is made for inclusion of some of the foregoing types of information with the publishing of the petition. However, no such provision has been made in Section 409 of the Act for food additives or in the food additives regulations pertaining to this Section.

Other Factors in Establishing Tolerances

Of significance perhaps to those dealing with petitions for food additives is the relationship of some statements made under Section 409 of the Act to certain regulations pertaining to this Section. Section 409(c)(4)(A), dealing with Action on the Petition, points out that, if a tolerance limitation is required to assure that the proposed use of an additive will be safe, the tolerance shall not be higher than the amount required to accomplish the physical or other technical effect for which such additive is intended. On the other hand, food additive regulation 121.5, dealing with safety factors, indicates that a safety factor in applying animal experimentation data to man of 100 to 1 will be used. That is, a food additive for use by man will not be granted a tolerance that will exceed $^1/_{100}$ of the maximum amount demonstrated to be without harm to experimental animals. Thus, on the one hand the residue levels obtained from feeding levels used to produce the desired physiological effect in the animal could be infinitesimally low with respect to the amount that could be tolerated based on the safety, yet the tolerance established would be based on the analytical data obtained on experimental samples rather than on a safe amount. For example, suppose an analytical method capable of sensitivity of 0.1 p.p.m. indicates no residue level in tissue of animals fed at the effective feeding level. This would suggest a zero tolerance be established by the FDA in accord with Section 409(c)(4)(A). Also, assume that 100 p.p.m. is perfectly safe for man when applying the statement under regulation 121.5. This would mean that, in this particular example, converting safety data from animal to man would result in a factor of something greater than 100,000 to 1. If it is further assumed that, in this particular situation, the true value of residue level lies in the upper portion of the sensitivity range of the analytical method, then one could expect that by chance alone, a residue would in all probability show up in actual widespread usage of the additive because of inherent variation in animal drug absorption and metabolism which might not have been reflected in the experimental population from which the test samples were withdrawn. It would therefore seem logical that, when establishing a tolerance for a residue where the safety data would permit, a more reasonable tolerance would be somewhat above the upper limit of the sensitivity range.

Importance of the Analytical Chemist in Residue Work

In obtaining a positive tolerance for an additive, long-term pharmacological and toxicity studies must be performed with the additive. Here the analytical chemist plays a role in establishing the fate of a compound being fed to a food-producing animal, or of a compound being used on an edible plant crop, prior to initiating long-term pharmacological studies. Is the material metabolized in the plant or animal? Is it perhaps translocated in the plant, or does it simply remain as the intact compound on the surface of the plant? This type of question should be answered at an early stage by the analytical chemist; otherwise it could be extremely embarrassing and costly to find that after 2 years of toxicity studies, the compound being tested is in fact not the compound really existing as the residue. In harvesting a test sample for the residue laboratory, it becomes extremely important to know size and number of test samples and control samples. The experiment can become meaningless unless the correct number of samples are taken, treated, and stored in a proper manner prior to submission to the laboratory. If the harvested samples cannot be analyzed immediately, the analytical chemist must provide sufficient information regarding storage requirements. Here the residue chemist must know something about the stability of the compound in the presence of tissue being studied. Can the tissue be frozen for a relatively long period of time without destroying the residue, or does it require a very brief storage due to the unstable nature of the compound in the tissue? One can attempt to answer such questions only by subjecting the additive to intimate contact with the test tissue under the appropriate storage condition over a reasonable period of time, and in this manner establish the stability of the additive in the test tissue.

End of Symposium

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