EXHIBIT A

TO DECLARATION OF
JENNIFER A. SORENSON

FDA 1977 Penicillin Notice

DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE

Food and Drug Administration

[Docket No. 77N-0230]

DIAMOND SHAMROCK CHEMICAL CO., ET AL.

Penicillin-Containing Premixes; Opportunity for Hearing

AGENCY: Food and Drug Administration.

ACTION: Notice.

SUMMARY: This is a notice of opportunity for a hearing on the proposal by the Director of the Bureau of Veterinary Medicine to withdraw approval of new animal drug applications (NADA's) for all penicillin-containing premixes intended for use in animal feed on the grounds that (1) new evidence shows that the penicillin-containing products have not been shown to safe for subtherapeutic use as required by section 512(e)(1)(B) of the Federal Food, Drug, and Cosmetic Act (21 U.S.C. 360b(e)(1)(B)) and § 558.15 (21 CFR 558.15); (2) the applicants have failed to establish and maintain records and make reports as required by section 512(e)(2)(A) of the act (21 U.S.C. 360b(e)(2)(A)) and § 558.15; and (3) new evidence shows that there is a lack of substantial evidence that penicillin-containing premixes are effective for therapeutic uses under section 512(e)(1)(C) of the act (21 U.S.C. 360b(e)(1)(C)).

DATES: Written appearances requesting a hearing must be submitted by September 29, 1977. Data and analysis upon which a request for a hearing relies must be submitted by October 31, 1977.

ADDRESS: Written appearances and data and analysis to the Hearing Clerk (HFC-20), Food and Drug Administration, Rm. 4-65, 5600 Fishers Lane, Rockville, Md. 20857.

FOR FURTHER INFORMATION CONTACT:

Gerald B. Guest, Bureau of Veterinary Medicine (HFV-130), Food and Drug Administration, Department of Health, Education, and Welfare, 5600 Fishers Lane, Rockville, Md. 20857 (301-443-3410).

SUPPLEMENTARY INFORMATION

RELATED ACTIONS

In a notice published elsewhere in this issue of the FEDERAL REGISTER, the Director of the Bureau of Veterinary Medicine is proposing to delete the provisions that provide for the use of penicillin in animal feeds by amending § 505.10 *Animal drug warning and caution statements required by regulations* (21 CFR 505.10); § 510.5 *Certification of new animal drugs containing any kind of penicillin, streptomycin, chlortetracycline, chloramphenicol, or bacitracin, or derivative thereof* (21 CFR 510.5); § 510.515 *Animal feeds bearing or containing new animal drugs subject to the provisions of section 512(n) of the act* (21 CFR 510.515); § 558.15 *Antibiotic, nitrofuran, and sulfonamide drugs in the feed of animals* (21 CFR 558.15); § 558.55 *Amprolium* (21 CFR 558.55); § 558.58 *Amprolium and ethopabate* (21 CFR 558.58); § 558.76 *Bacitracin methylene disalicylate* (21 CFR 558.78); § 558.78 *Bacitracin, zinc* (21 CFR 558.78); § 558.105 *Buquinolate* (21 CFR 558.105); § 558.145 *Chlortetracycline, procaine penicillin and sulfamethazine* (21 CFR 558.145); § 558.155 *Chlortetracycline, procaine penicillin and sulfathiazole* (21 CFR 558.155); § 558.274 *Hygromycin B* (21 CFR 558.274); § 558.460 *Penicillin* (21 CFR 558.460) § 558.530 *Roxarsone* (21 CFR 558.530); and § 558.680 *Zoalene* (21 CFR 558.680).

DISCUSSION

Since the Director's discussion of the issues involved in this matter is necessarily detailed, he is setting forth, for the reader's convenience, an outline of the discussion as follows:

I. THE DRUG

Name. Procaine penicillin G (benzylpenicillin) or feed grade penicillin, alone or in combination with other drugs.

Dosage form. Feed premix.

Approvals. The following companies hold or have effective approvals that are covered by this notice:

NADA 39-077; CSP 250 (chlortetracycline, sulfathiazole, and procaine penicillin); Diamond Shamrock Corp., 1100 Superior Ave., Cleveland, OH 44114.

NADA 35-688, Aureo SP-250 Feed Premix (Chlortetracycline, sulfamethazine, and procaine penicillin); American Cyanamid Co., P.O. Box 400, Princeton, NJ 08540.

NADA 46-667; Micro-Pen and Streptomycin Sulfate Premixes, (procaine penicillin G and streptomycin sulfate). Micro-Pen 6.25 and Streptomycin Sulfate 18.75, Micro-Pen and Streptomycin Sulfate 75, Micro-Pen and Streptomycin Sulfate 45, Micro-Pen and Streptomycin Sulfate 150; Elanco Products Co., Division of Eli Lilly Co., Indianapolis, IN 46206.

DESI 0072NV; Micro-Pen and MicroPen 100 (procaine penicillin G); Elanco Products Co.

NADA 35-207; Amprolium, Ethopabate and Penicillin; Merck, Sharp & Dohme Research Laboratories, Division of Merck & Co., Inc., Rahway, NJ 07065.

NADA 46-593; Pro-Pen 50% Penicillin Mixture Medicated, Pro-Pen "20" Penicillin Mixture Medicated, Pro-Pen 90% Penicillin Mixture Medicated, and Pro-Pen "100" Penicillin Mixture Medicated; Merck, Sharp & Dohme Research Laboratories.

NADA 9-476; Nicarbazin, Penicillin with/or without Roxarsone; Merck, Sharp & Dohme Research Laboratories.

NADA 46-981 Pro-Strep (procaine penicillin, streptomycin sulfate); Merck, Sharp & Dohme Research Laboratories.

NADA 46-726; Streptomycin and Procaine Penicillin Premix 15+5, Streptomycin and Procaine Penicillin Premix 18.75+6.25, Streptomycin and Procaine Penicillin Premix 45+15, Streptomycin and Procaine Penicillin Premix 75+25; Pfizer, Inc., New York, NY 10017.

NADA 46-668; Penicillin Premix P-4, Penicillin Premix P-50, and Penicillin Premix P-100; Pfizer, Inc.

NADA 49-287; Chlorachel 250-Swine (chlortetracycline, sulfamethazine, and procaine penicillin G): Rachelle Laboratories, Inc., 700 Henry Ford Ave., P.O. Box 2029, Long Beach, CA 90801.

NADA 91-668; Super Chlorachel 250-Swine (chlortetracycline, sulfamethazine, and procaine penicillin G); Rachelle Laboratories, Inc.

NADA 46-666; Penicillin G Procaine for Animal Feeds 50 percent and Penicillin G Procaine for Animal Feeds 100 percent; E. R. Squibb & Sons, Inc., P.O. Box 4000, Princeton, NJ 08540.

Under section 108(b)(2) of the Animal Drug Amendments of 1968 (Pub. L. 90-399), any approval of a new animal drug granted prior to the effective date of the amendments whether through approval of a new drug application, master file, antibiotic regulation, or food additive regulation, continues in effect until withdrawn in accordance with the provisions of section 512 of the Federal Food, Drug, and Cosmetic Act (21 U.S.C. 360b). Many such approvals were issued long ago, and some may never have been used by the holder of the approval. Consequently, the current files of the Food and Drug Administration (FDA) may be incomplete and may fail to reflect the existence of some approvals. Also, many approvals have been withdrawn by other agency actions, e.g., FDA's rulemaking procedure published in the FEDERAL REGISTER of February 25, 1976 (41 FR 8282). The burden of coming forward with documentation of unrecorded approvals in such circumstances is therefore properly placed on the person claiming to hold such approvals so as to permit definitive revocation or amendment of the regulations.

The Director of Bureau of Veterinary Medicine knows of no approvals affected by this notice other than those named herein. Any person who intends to assert or rely on such an approval that is not listed in this notice shall submit proof of its existence within the period allowed by this notice for opportunity to request a hearing. The failure of any person holding such an approval to submit proof of its existence within that period shall constitute a waiver of any right to assert or rely on it. In the event that proof of the existence of such an approval is presented, this notice shall also constitute a notice of opportunity for hearing with respect to that approval, based on the same grounds set forth in this notice.

Conditions of use. All uses of penicillin in penicillin and penicillin-containing combination drug products as cited in:

Sections 510.515, 558.15, 558.55, 558.58, 558.76, 558.78, 558.105, 558.145, 558.155, 558.274, 558.460, 558.530 and 558.680.

II. INTRODUCTION

A. *Regulatory Background*

Antibacterial drugs have been used at subtherapeutic levels (lower levels than therapeutic levels needed to cure disease) in animal feed for over 25 years. Growth benefits from this use were first observed when animals were fed the discard products from the fermentation process that was originally used in the manufacture of chlortetracycline. The precise mechanism of action, however, remains unclear.

Initially, certifiable antibiotics for use in animal feed such as penicillin were regulated under the provisions of section 507 of the Federal Food, Drug, and Cosmetic Act (21 U.S.C. 357). Unlike the basic private licensing system applicable to new drugs, the provisions of section 507 created a public regulation or monograph system for regulating these products, in part because of the complexities in manufacturing the products and the lack of knowledge of their chemical structures. Antibiotic residues in food from food-producing animals were then regulated under the provisions of the act dealing with adulteration and misbranding. After enactment of the Food Additives Amendment of 1958 (Pub. L. 85-929), however, residues were principally regulated by section 409 of the act (21 U.S.C. 349), which also established a public monograph system of premarket approval. Under the antibiotic monograph procedure, the pioneer manufacturer generated and submitted the basic safety and effectiveness data in an FD Form 5 (now FD-1675). A regulation was subsequently published setting forth the standards of identity, strength, quality, and purity and the packaging and labeling requirements that the product must meet. FDA approval of the same product made by another manufacturer was then conditioned solely upon a demonstration that it met the requirements of the regulation, and this is normally accomplished by batch certification. Section 507(c) of the act (21 U.S.C. 357(c)), however, permits the agency to exempt by regulation any drug or class of drugs from the certification requirement when it concludes that certification is unnecessary for the manufacture of the drugs. Antibiotics for use in animal feeds as feed ingredients were exempted from the certification requirements in 1951 (see the FEDERAL REGISTER of April 28, 1951 (16 FR 3647)), and those for use as drugs were exempted in 1953 (see the FEDERAL REGISTER of April 22, 1953 (18 FR 2335)). These are now set out in §§ 510.510 and 510.515 (21 CFR 510.510 and 510.515).

Congress enacted the Animal Drug Amendments of 1968 (Pub. L. 90-399) and consolidated the provisions of the act dealing with the premarket approval of drugs intended for use in animals (sections 409, 505, 507) into one new section, section 512 (21 U.S.C. 360b), to regulate these articles more efficiently and effectively (Senate Committee on Labor and Public Welfare, Animal Drug Amendments of 1968, S. Rep. No. 1308, 90th Cong., 2d Sess. (1968)). This legislation also brought the manufacture of antibiotics under the private license system for new drugs (id.; Hearing on S. 1600 and H.R. 3639 Before the Subcommittee on Health of the Senate Committe on Labor and Public Welfare, 90th Cong., 2d Sess. (1968)). To efficiently accomplish this change, the amendments contained a transition clause (section 108 (b)) which provided that all prior approvals continue in effect and be subject to change in accordance with the provisions of the basic act as amended. In summary, all persons legally marketing antibiotics under the provisions of sections 409, 505, and 507 of that act on August 1, 1969, the effective date of the Animal Drug Amendments of 1968, were considered as holding the equivalent of an approved new animal drug application; however, all holders of such approvals are also subject to all applicable requirements of the act and regulations.

B. *Safety Concerns*

In the mid-1960's, FDA became concerned about the safety to man and animals of subtherapeutic antibiotic use; it studied the effects of low-level subtherapeutic feeding of antibiotics for some years. The agency supported research, held symposia, and consulted with outside experts to review these nonmedical uses of antibiotics in animal feeds. Following a report issued by the British Government Joint Committee (the Swann Committee) "On the Use of Antibiotics in Animal Husbandry and Veterinary Medicine," the Commissioner of Food and Drugs in April 1970 established a Task Force of scientists, with consultants from government, universities, and industry, to review comprehensively the use of antibiotic drugs in animal feeds. Its conclusions were published in a notice of proposed rule making published in the FEDERAL REGISTER of February 1, 1972 (37 FR 2444), which initiated the mandatory testing procedure to resolve conclusively the issues of safety surrounding the subtherapeutic use of antibiotics in animal feeds.

The principal conclusions of the Task Force were the following: (1) The use of antibiotics and sulfonamide drugs, especially in growth promotant and subtherapeutic amounts, favors the selection and development of single and multiple antibiotic-resistant and R-plasmid-bearing bacteria;

(2) Animals that have received either subtherapeutic and/or therapeutic amounts of antibiotic and sulfonamide drugs in feeds may serve as a reservoir of antibiotic resistant pathogens and nonpathogens. These reservoirs of pathogens can produce human infections.

(3) The prevelance of multiresistant R-plasmid-bearing pathogenic and nonpathogenic bacteria in animals has increased and has been related to the use of antibiotics and sulfonamide drugs.

(4) Organisms resistant to antibacterial agents have been found on meat and meat products.

(5) There has been an increase in the prevalence of antibiotic- and sulfonamide-resistant bacteria in man.

In its report to the Commissioner, the Task Force also identified three areas of primary concern: Human health hazards, animal health hazards, and antibiotic effectiveness; and guidelines were established to show whether use of any antibiotic or antibacterial agent in animal feed presents a hazard to human and animal health.

The February 1972 proposal also announced that all currently approved subtherapeutic uses of antibiotics, nitrofurans, and sulfonamides in animal feeds would be revoked unless data were submitted to resolve conclusively the issues concerning safety to man and animals in accordance with the Task Force guidelines. That notice also proposed to establish a time table for filing commitments, conducting studies, and submitting relevant data and information. Based on the guidelines, the agency then began developing specific criteria by which safety and effectiveness of each antibiotic product might be established. The notice further suggested that protocols be submitted to the agency for comment. The criteria and studies to address them may be summarized as follows:

HUMAN AND ANIMAL HEALTH SAFETY CRITERIA

1. Transfer of drug resistance: (a) An antibacterial drug fed at subtherapeutic levels to animals must be shown not to promote increased resistance to antibacterials used in human medicine. Specifically, increased multiple resistance capable of being transferred to other bacteria in animals or man should not occur. (b) If increased transferable multiple resistance is found in coliforms, studies may be done to show whether this resistance is transferable to man.

2. The *Salmonella* reservoir: The use of antibacterial drugs at subtherapeutic levels in animal feed must be shown not to result in (a) an increase in quantity, prevalence or duration of shedding of Salmonella in medicated animals as compared to nonmedicated controls; (b) an increase in the number of antibiotic resistant Salmonella or in the spectrum of antibiotic resistance; (c) disease (caused by Salmonella or other organisms) that is more difficult to treat with either the same medication or other drugs.

3. The use of subtherapeutic levels of an antibacterial drug should not enhance the pathogenicity of bacteria, e.g., by increasing enterotoxin production. The association of toxin production characteristics with transfer factors must be investigated in well-designed studies. (Final resolution of this question was not expected within the 2-year period. Drug sponsors were expected to show evidence of work underway which would lead toward answers to this question.)

4. An antibacterial drug used at subtherapeutic levels in the feed of animals shall not result in residues in food ingested by man which may cause either increased numbers of pathogenic bacteria or an increase in the resistance of pathogens to antibacterial agents used in human medicine. Hypersensitivity to residues was to be addressed by a literature survey.

The Commissioner promulgated a final order that was published in the FEDERAL REGISTER of April 20, 1973 (38 FR 9811), and at that time the requirements imposed by the regulation became legally binding on all firms marketing antibacterial drugs used at subtherapeutic levels in feed. In the FEDERAL REGISTER of August 6, 1974 (39 FR 2839), the Commissioner proposed withdrawal of all approvals held by persons who had not complied with the initial requirements, and all these approvals were withdrawn by his order, published in the FEDERAL REGISTER of February 25, 1976 (41 FR 8282). Therefore, only those products listed in Part 558 (21 CFR Part 558) can be legally marketed at this time.

By April 20, 1974, the Bureau of Veterinary Medicine (Bureau) had begun a review of the data required by § 558.15 which was applicable to the principal antibiotics used subtherapeutically in animal feeds (penicillin and tetracycline), and by April 20, 1975, data concerning the safety and efficacy criteria for all antibiotic and sulfonamide drugs had been received. To assist the Bureau, the Commissioner asked the agency's National Advisory Food and Drug Committee (NAFDC) to review the data and issues involved and to make recommendations to him on the future uses of subtherapeutic antibiotics in animal feeds. A subcommittee of three members, the Antibiotics in Animal Feeds Subcommittee (AAFS), was appointed to work in conjunction with four expert consultants from disciplines related to the issue. The Bureau prepared 2 days' presentations concerning penicillin during which comments were heard from the drug industry, animal scientists, and other interested parties. The Bureau also prepared a comprehensive summary report with tentative recommendations for the subcommittee. (An identical procedure was carried out for the tetracyclines.) Two additional meetings were held during which subcommittee deliberations were conducted and other statements given.

In September 1976, the AAFS presented its preliminary recommendations to the parent NAFDC, and in January 1977, the subcommittee's final report was submitted to the NAFDC. The parent committee reviewed the recommendations on penicillin and accepted them. NAFDC recommended that FDA immediately withdraw approval for the subtherapeutic uses of penicillin, i.e., growth promotion/feed efficiency, and disease control.

In view of these recommendations and since the information submitted in response to § 558.15 following the guidelines and criteria had failed to resolve conclusively the issues of safety concerning subtherapeutic uses of penicillin in animal feeds, the Director of the Bureau of Veterinary Medicine is therefore proposing to withdraw approval of all subtherapeutic uses of penicillin alone and in combination with other drugs in animal feeds. Because the National Academy of Sciences/National Research Council Drug Efficacy Study Group concluded that the therapeutic use of penicillin in animal feed lacked substantial evidence of effectiveness, he is also proposing to withdraw approval of all penicillin use in animal feed.

III. SUMMARY OF THE ARGUMENT

Soon after his advisory of penicillin, Sir Arthur Fleming noted that some bacterial organisms could become resistant to the antibiotic. As the use of antibiotics has increased, the number and types of bacterial resistance have also multiplied. There is a serious concern that, in time, this will lead to declining usefulness of antibiotics in the treatment of both human and animal diseases.

The Bureau's primary concern is with that portion of increased antibiotic resistance in the ecological system which may result from the practice of using subtherapeutic levels of penicillin and other antibiotics in animal feed for prolonged periods. This practice, which sometimes produces increases in growth promotion/feed efficiency, provides an ideal environment for selective pressure to operate. When exposed to an antibiotic, the organisms that are drug resistant survive while the growth of other (drug-sensitive) bacteria is inhibited. Eventually, the antibiotic-resistant organisms predominate in the bacterial population, and continuous antibiotic pressure perpetuates this abnormal situation.

Bacterial antibiotic resistance is primarily determined by genetic elements termed "R-plasmids" (R-factors, R+). The Bureau's specific concern, therefore, is with the health hazard that may arise through an increase in the pool of R-plasmids in the animal population and the potential transfer of these R-plasmids and R-plasmid-bearing organisms to the human population and surrounding environment.

R-plasmids are small lengths of DNA that are separate from the bacterial chromosome. These R-plasmids carry transferable genes for drug resistance as well as the capacity to reproduce themselves. Plasmids may determine resistance to more than one antibiotic, and resistance to several antibiotics is common. Moreover, plasmids can transfer from one bacteria to another and from nonpathogenic to pathogenic strains. Transfer occurs, although with varying frequency among all members of the enteric bacteria and also to members of other families of bacteria. The pool of normal Gram-negative bacterial intestinal flora (largely *Escherichia coli*) serves as a reservoir of R-plasmids; the R-plasmid-bearing bacteria interchange among animals, man, and the environment. The potential for harm increases as the R-plasmid reservoir increases because the probability of R-plasmid transfer to pathogens increases. When the Commissioner required all holders of approved NADA's for the subtherapeutic use of penicillin in animal feed to submit data to resolve the safety questions raised, he was principally concerned with the effect of the antibiotics approved for subtherapeutic use in animal feed on the emergence of transferable drug resistance in the Salmonella reservoir and the *E. coli* of animals. In the Director's opinion, the results of the studies submitted and the data available are clear; the affected parties have failed to answer the safety questions raised.

Evidence demonstrates that the use of subtherapeutic levels of penicillin and other antibiotics in animal feed contributes to the increase in antibiotic resistant *E. coli* and in the subsequent transfer of this resistance to Salmonella. Further, many strains of *E. coli* and Salmonella infect both man and animals.

The holders of approved NADA's have submitted no evidence to demonstrate that the observed strains of *E. coli* and Salmonella in man and animals are mutually exclusive; in fact, the evidence is overwhelming to the contrary. Furthermore, in some cases the R-plasmids as well as the resistance genes from humans and animal sources are indistinguishable. Thus, the potential for harm exists, as illustrated by the studies submitted and verified by evidence from studies conducted by independent scientists. No evidence has been submitted by any NADA holder to resolve conclusively the safety questions raised by this potential in accordance with the requirements of § 558.15.

The holders of approved NADA's were also required to submit studies demonstrating that the subtherapeutic use of penicillin in animal feed would not compromise subsequent antibiotic therapy in man or animals, but animal studies submitted on their behalf by the Animal Health Institute to determine whether subtherapeutic penicillin use compromised subsequent therapy with related drugs were inconclusive because the studies were inproperly designed. Thus, holders also failed to show conclusively that subtherapeutic penicillin use is safe in accord with that criterion.

Additionally, the NADA holders were required to prove that the subtherapeutic use of penicillin would not increase the pathogenicity of the infecting organism. They have submitted no adequate studies on the issue, and other recent evidence now suggests that the genetic determinants for toxic production may become linked with drug resistance genes. Thus, the sponsors failed to satisfy that criterion also.

No studies have ever been submitted on the issues of the safety of penicillin residues in food or the effect of long-term use on the penicillin levels needed to maintain their subtherapeutic effectiveness.

Finally, the National Academy of Sciences/National Research Council Drug Efficacy Study Group evaluated the effectiveness claims for the penicillin premixes and concluded that there was a lack of substantial evidence that the premixes were effective for their therapeutic claims. No adequate and well-controlled investigations showing that these products are effective have been submitted.

None of the specified human and animal health safety criteria have been satisfied, and the premixes lack substantial evidence of effectiveness for their therapeutic claims. For all the foregoing reasons, the Director is proposing to withdraw approval of all NADA's for the use of penicillin and combination products, e.g., chlortetracycline-sulfamethazine-penicillin, in animal feed.

IV. STUDIES RELEVANT TO HUMAN AND ANIMAL HEALTH SAFETY CRITERIA

A. *Transfer of Drug Resistance (Criterion 1). The Pool of R-Plasmid-Bearing Organisms is Increasing*

1. *Background.* One of the most important animal and human health safety criteria (number 1., set forth in II. B. above) concerns the role of subtherapeutic antibiotic use on the selection for and increase in the pool of microbial plasmids determining multiple drug resistance, and in the transfer of these plasmids among bacteria in animals and man. Resistance to antibiotics has been known as long as the antibiotics themselves have been known. Until 1959 it was believed that antibiotic resistance was a result of chance mutation and natural selection alone. However, in 1959, Japaneses investigators (Ref. 1) discovered that resistance to several common antimicrobial agents could be transferred simultaneously from one bacterium to another by cell-to-cell contact (conjugation). This was shown to be due to the transfer of extrachromosomal resistance determinants called R-plasmids, i.e., R-factors, or R+. Resistance produced by R-plasmids generally involves the production of enzymes that inactivate the antibiotic. For example, R-plasmid mediated penicillin resistance is due to the production of an enzyme, penicillinase, that inactivates the penicillin molecule. This same enzyme is also active against many semisynthetic penicillins, including ampicillin. R-plasmids may carry as many as nine drug resistance genes. The plasmids also carry other genes that determine the R-plasmid's replication, independent of the host chromosome, as well as information for transfer of the R-plasmids from one bacterium to another by conjugation. R-plasmids are transferred by conjugation to virtually all Enterobacteriaceae as well as to such unrelated Gram-negative bacteria as *Vibrio, Pseudomonas,* and *Pasteurella.* Thus, resistance may pass from strain to strain, species to species, and most importantly, from nonpathogen to pathogen. R-plasmids are now known to be the predominant cause of antibiotic resistance in Gram-negative organisms that cause human disease, e.g., *E. coli, Salmonella, Shigella,* etc.

While the development of antibiotics revolutionized the treatment of infectious disease in both man and animals, the magnitude of this achievement has been diminished by the widespread emergence of antibiotic resistant bacteria. R-plasmid mediated resistance is particularly ominous since selection of resistance to a single antibiotic may also lead to the simultaneous selection of resistance to a wide spectrum of other antibiotics. In recent years, antibiotic resistance has emerged in important pathogens; for example, in *Haemophilus, Neisseria gonorrhoeae,* and *Salmonella typhi.* R-plasmid mediated resistance has been identified in epidemics around the world, e.g., *Salmonella typhimurium.* Some of these organisms have acquired both ampicillin and chloramphenicol resistance, producing disease that will no longer respond to therapy. Hence, drug-resistant organisms have become an important concern in both human and veterinary medicine. (Ref. 2 and 3).

Because the use of antibiotics is extensive, an effort must be made to assure the future utility of these lifesaving products. In 1960, the annual production of antibiotics in the United States was 4.16 million pounds, of which 2.96 million pounds were used for therapeutic purposes in human and veterinary medicine and 1.20 million pounds in animal feed additives. By 1970, 9.6 million pounds were being used for human and veterinary medicine pharmaceuticals; for animal feed additives, 7.3 million pounds were being used. Moreover, according to "Synthetic Organic Chemicals, United States Production and Sales (1971–1975)" (U.S. International Trade Commission Publication 804), the 5-year average production for 1971 through 1975 was 11.16 million pounds for medicinal uses and 7.68 million pounds for nonmedicinal uses, including feed additive uses. Over those 5 years, the aggregate average of the total production for those nonmedicinal uses was 40.8 percent—but 48.6 percent in 1975. Thus the use of antibiotics in animal feeds is a considerable element in the overall use of antibiotics in this country and consequently must be considered a potentially significant contributor to the resistance problem.

REFERENCES

1. Watanabe, R., "Infective Heredity of Multiple Drug Resistance in Bacteria," Bacteriological Reviews, 27:87–115, 1963.

2. Simmons, H. and P. D. Stolley, "This is Medical Progress?" Journal of the American Medical Association, 222:1023–1028, 1974.

3. Linton, A. H., "Antibiotic Resistance: The Present Situation Reviewed," Veterinary Record, 100:354–360, 1977.

2. *Criterion.* The FDA Task Force concluded that a human health hazard exists if the subtherapeutic use of antibiotics in animal feeds leads to an increase in R-plasmid-bearing organisms, if these antibiotics used subtherapeutically are also used in human clinical medicine, and if R-plasmids subsequently appear in bacteria in man. It was the intent of the Task Force as well as the intent of § 558.15 to reduce the total load of resistant organisms in the environment and to ensure the effectiveness of antibiotics in the treatment of disease in man and animals. Accordingly, § 558.15 required the following:

An antibacterial drug fed to animals shall not promote an increase of coliforms that are resistant to antibacterial drugs used in human clinical medicine and capable of transferring this resistance to bacteria indigenous to the intestinal tract of man. Studies must be undertaken to assess the occurrence and significance of these events:

a. Controlled studies shall be undertaken to determine whether or not the administration of an antibacterial drug at low and/or intermediate levels to target animals results in an increase in the numbers of coliforms bearing R-

plasmids present in the intestinal tract of the animal or a change in the resistance spectrum of these organisms compared to those found in controls receiving no antibacterial drug. The resistance spectrum must be determined to ascertain whether or not there are determinants present for resistance to antibacterial drugs used in human clinical medicine.

b. If the resistance determinants indicated in a. are found, a sponsor may elect to conduct additional studies to determine if such multiple drug resistance is transferable to the indigenous coliforms in the intestinal tract of man.

In addition to the FDA Task Force, many other scientists were concerned that the use of antibiotics at subtherapeutic levels in feed might lead to the development of R-plasmid-bearing organisms in food animals, which might then spread to man. The normal enteric organisms that can serve as this reservoir are the coliforms, in particular *E. coli*. These *E. coli* can donate the R-plasmids to other bacteria, including pathogens, e.g., pathogenic *E. coli, Salmonella,* etc.

3. *Studies Relevant to Transfer of Drug Resistance*—(a) *R-plasmid-bearing E. coli develop in domestic animals that are fed subtherapeutic levels of antibiotics, including penicillin.* Many investigators have reported the presence of R-plasmid-bearing *E. coli* in domestic animals such as chickens, swine and cattle. The influence of antibiotic-supplemented feed in increasing the number of resistant organisms has been extensively documented. Mercer et al. (Ref. 1) showed that 394 of 491 isolates (80 percent) from animals exposed to antibiotics in feed, including penicillin, were resistant strains, and in contrast, only 14 of 64 isolates (21.9 percent) obtained from animals not exposed to antibiotics in feed were resistant strains. Mercer also reported that plasmid-mediated ampicillin resistance occurred more frequently in animals that were exposed to subtherapeutic levels of penicillin in their feed than in nonmedicated animals. Seigel et al. (Ref. 2) Smith and Tucker (Ref. 3), Katz et al. (Ref. 4), and others have also shown that the addition of penicillins to feed at subtherapeutic levels causes a significant increase in the R-plasmid-bearing coliform population of the intestinal flora of animals. Even the data submitted by the drug industry on the effect of subtherapeutic use of penicillin on the *E. coli* flora of poultry, which will be discussed in depth in part IV. B. 3. below, also show an increase in drug-resistant *E. coli*.

Accordingly, the Director has concluded that subtherapeutic use of penicillin in animal feed produces a high level of antibiotic resistant *E. coli* and that the subtherapeutic use of penicillin selects for R-plasmid-containing bacteria in animals (human health criteria 1.(a) set forth in II. B. above), i.e., the antibiotic pressure of subtherapautic penicillin use allows microbial R-plasmid-containing populations to predominate. These populations appear to be stable and persistent, even in the absence of penicillin pressure. Once the reservoir of R-plasmids develops, the initial cause of the R-plasmid buildup, whether the subtherapeutic use of penicillin or another drug (or drug combinations), is irrelevant to the R-plasmids' transferability or movement from animals to humans. Therefore, all studies on the movement of R-plasmids and resistant bacteria are germane to this issue even though penicillin was not always used as the specific antibiotic.

REFERENCES

1. Mercer, H. D., D., Pocurul, S. Gaines, S. Wilson, and J. V. Bennett, "Characteristics of Antimicrobial Resistance of *Escherichia coli* from Animals: Relationship to Veterinary and Management Uses of Antimicrobial Agents," Applied Microbiology, 22:700–705, 1971.

2. Seigel, D., W. G. Huber, and F. Enloe, "Continuous Nontherapeutic Use of Antibacterial Drugs in Feed and Drug Resistance of the Gram-negative Enteric Flora of Food-Producing Animals," Antimicrobial Agents and Chemotherapy, 6:697–701, 1974.

3. Smith, H. W., and J. F. Tucker, "The Effect of Antibiotic Therapy on the Faecal Excretion of *Salmonella typhimurium* by Experimentally Infected Chickens," Journal of Hygiene, 75:275–292, 1975.

4. Katz, S. E., C. A. Fassbender, P.S. Dinnerstein, and J. J. Dowling, Jr., "Effects of Feeding Penicillin to Chickens," Journal of the Association of Official Analytical Chemists, 57:522–526, 1974.

(b) *E. coli contribute their R-plasmids to man through several mechanisms.* There has been much debate over the extent to which *E. coli* in the animal community act as a source of R-plasmid-bearing strains for man. This is perhaps the most controversial and most difficult aspect of R-plasmid ecology to assess. Drug-resistant bacteria originating in animals may reach man (1) by direct contact with animals, (2) through the food chain, and (3) because of their widespread occurrence in the environment.

(i) Direct contact with animals: A number of studies have shown that humans in contact with animals receiving medicated feed, including subtherapeutic levels of penicillin, have a higher incidence of drug-resistant organisms in their flora than do control populations without this direct contact. Linton et al. (Ref. 1) found a higher incidence of drug-resistant *E. coli* in adults employed with livestock husbandry than other rural or urban adults. Wells and James (Ref. 2) found a higher incidence of drug-resistant *E. coli* in humans in contact with pigs given certain antibiotics than in humans in contact with pigs that had not been given antibiotics.

Seigel et al. (Ref. 3) compared the proportion of resistant organisms in fecal samples from: (*a*) people working on farms who were continuously in contact with the predominantly resistant flora of animals receiving subtherapeutic levels of penicillin; (*b*) people residing on the same farms with no direct exposure to the farm animals; (*c*) people treated with antibacterial drugs; (*d*) untreated people residing with treated individuals; (*e*) untreated people with no exposure to farm animals or treated individuals.

The data (Ref. 3) indicate that the enteric flora of individuals not directly exposed to the selective effects of antibiotics can be affected by contact with animals; furthermore, these individuals may be affected by contact with other people who have a predominantly resistant flora as a result of their exposure to subtherapeutic levels of antibacterials in feeds.

A study sponsored by the Animal Health Institute, Levy et al. (Ref. 4), examined the change in intestinal microflora of chickens, farm dwellers, and their neighbors before and after a tetracycline-supplemented feed was introduced on the farm. Within 1 week after introduction of this antibiotic in their diet, the *E. coli* of the chickens were almost entirely tetracycline resistant. Subsequently, at a slower rate, increased numbers of antibiotic-resistant bacteria appeared in the flora of the farm dwellers. No such increase was observed in the farm neighbors, who were not exposed to the animals fed subtherapeutic antibiotics. Within 5 to 6 months, 31.3 percent of weekly fecal samples from farm dwellers contained greater than 80 percent tetracycline-resistant bacteria compared to 6.8 percent of the samples from the neighbors. This is statistically significant ($P<0.001$). Using a specially marked resistance gene to identify a particular plasmid, Levy was also able to demonstrate the direct spread of resistant organisms from chickens to chickens and from chickens to man (Ref. 5).

Although penicillin was not used in this study, resistance to both penicillin and tetracycline is plasmid mediated; therefore, the study is germane to the question of the transfer of resistant organisms from animals to man. These studies demonstrate that the subtherapeutic use of certain antibiotics increases the pool of R-plasmid-bearing *E. coli*, and they define one route by which antibiotic-resistant strains can enter the human population. While this route is of great importance to farm dwellers, the majority of the population has no contact with live animals. For this segment of individuals, a more important route of exposure by which resistant bacteria can pass to man is by the handling and ingestion of meat and poultry products contaminated with R-plasmid-bearing *E. coli* of animal origin.

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(ii) Contact with *E. coli*-contaminated food: To assess adequately the significance of the problem of human food contaminated with *E. coli*, Howe and Linton (Ref. 1) described four factors that must be measured: (*a*) The incidence of R-plasmid-bearing *E. coli* in food-producing animals; (*b*) the load and frequency of excretion of *E. coli* from these animals; (*c*) the degree and source of contamination of carcasses at slaughter; and (*d*) the overlap of *E. coli* serotypes in various host animals with those commonly found in humans. A number of surveys have clearly documented that pigs, calves, and poultry carry a large reservoir of antibiotic-resistant *E. coli*. These include investigations by Anderson; Loken; Mercer; Smith; Howe, Linton and Osborne; Smith and Crabb (Refs. 2 through 8, and 15). In these surveys, animals excrete large numbers of *E. coli* organisms resistant to a wide range of clinically useful antibiotics, and these animals clearly constitute a reservoir "rich" in R-plasmids. Moreover, they excrete a large variety of distinct serotypes of *E. coli*.

During the slaughtering process, contamination of carcasses with intestinal microorganisms cannot be prevented. Meat and meat products are often contaminated with antibiotic-resistant *E. coli*, and these often reach the human consumer. Walton (Ref. 9) demonstrated that 52 percent of the bovine (beef) and 83 percent of porcine (pork) carcasses slaughtered at commercial abattoirs were contaminated with *E. coli*. Walton and Lewis (Ref. 10) isolated resistant *E. coli* from 21 of 50 specimens of fresh meat and from 4 of 50 specimens of cooked meat. Babcock et al. (Ref. 11) isolated multiresistant *E. coli* from 80 percent of 98 samples of dressed beef. Resistance in most cases was found to be transmissible.

Similar incidents of *E. coli* contamination occur with the slaughter of chickens. Kim and Stephens (Ref. 12) found a high incidence of R-plasmid-bearing *E. coli* in "ready to cook" broiler chickens. The greatest number of *E. coli* isolated were obtained from the fluid and abdominal cavity, suggesting that the principal source of these microorganisms is the intestines. Furthermore, poultry meat has been incriminated as a source of *E. coli* for patients in hospitals (Cooke et al., and Shooter et al. (Refs. 14 and 18)).

The presence of antibiotic-resistant (R-plasmid-bearing) *E. coli* in the animal intestinal tract and on the carcass does not conclusively prove that the *E. coli* are identical organisms. However, recent studies using serotyping methods have characterized resistant and sensitive *E. coli* isloated from the animal intestinal tract and carcass (Refs. 13, 15, 16, and 17) and have found that the resistant O-serotypes on the carcasses of pigs, calves, and poultry frequently are identical to those isolated from the fecal contents of the same animal. Moreover, Linton, Howe, et al. (Ref. 17) showed that a large number of *E. coli* found on table-ready thawed chickens were resistant to therapeutically important antibiotics. The organisms reaching the kitchen included a wide diversity of O-serotypes of antibiotic-resistant *E. coli*. Similarly, Shooter et al. (Ref. 13) described the distribution and serotype of strains of *E. coli* from a poultry packing station and an abattoir. Shooter concluded that "results in both the abattoir and the poultry packing station indiacte that there is transfer of strains from the faeces of the animals to the environment and that the strains of *E. coli* found on the carcasses of poultry, cattle and beef will originate from the feces of the animal and from the environment and will reffect the history of the carcass."

Foodborne *Salmonella* infections in man are a well-recognized and continuing problem. Animal meat products that serve as a primary source of *Salmonella* infections in humans also serve as a source of other bacteria for man including R-plasmid-bearing enteric bacteria (Ref. 19). Based on this evidence, the Director must conclude that man is exposed to R-plasmid-bearing intestinal bacteria through contact with contaminated food. Because the drug resistance of these bacteria is increased by feeding the animals subtherapeutic levels of antibiotics, such feeding enhances the likelihood of transmitting R-factor-bearing bacteria to man through contact with contaminated food.

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(iii) Widespread presence in the environment: Many studies (Ref. 1 through 6) have shown that intestinal bacteria (e.g., *E. coli* and *Salmonella*) carrying R-plasmids are widespread in the environment. Resistant strains reach the environment from raw and treated municipal, hospital, and animal wastes. The number of R-plasmid-bearing bacteria reported in sewage and the effects of sewage treatment vary. Most surveys indicate that hospital sewage contains more drug-resistant coliforms, more R-plasmids, and a greater proportion of R-plasmids carrying multiple resistance than sewage from domestic and other sources. However, hospitals do not constitute a large proportion of total sewage. Therefore, Linton et al. (Ref. 4) compared the contributions of hospital and domestic sources to the total pooled sewage output of the city of Bristol, and they concluded that industrial and domestic sources, rather than the hospital population, appear to be by far the greatest contributors to the reservoir of R-plasmids in the community (Ref. 7).

R-plasmid-containing bacteria also occur in rivers and sea water, and some authors have urged stricter control of discharges to surface waters. Feary et al. (Ref. 2) examined the incidence of antibiotic-resistant *E. coli* present at sites along a fresh water river system and within the salt water bay into which it empties. Antibiotic-resistant coliforms were detected in nearly all the fresh water sites sampled and in about 50 percent of the salt water sites. Feary found that 20 percent of the 194 strains tested contained R-plasmids carrying multiple antibiotic resistance which could be transferred to sensitive *Salmonella typhimurium, Shigella dysenteriae,* and *E. coli.* They also isolated coliforms containing R-plasmid carrying resistance to chloramphenicol. Transferable chloramphenicol resistance is a significant health concern since chloramphenicol is often the antibiotic of choice for the treatment of typhoid fever. In Feary's study, the incidence of coliform organisms appeared higher around heavily populated areas, but coliforms were also recovered with ease from rural areas. In one case where particularly high counts were obtained, the sample was taken below a large cattle feedlot.

The high levels of resistant coliforms may be of more consequences in the salt water since certain sections are utilized heavily by fishermen in harvesting fish, shrimp, clams, and oysters. Oysters and clams are of primary concern because they continuously filter water and concentrate bacteria in their gut and they are often eaten uncooked.

Recent reports by Cooke (Ref. 1) have also described a high incidence of resistant coliforms in marine shellfish and freshwater mussels.

Therefore, the Director must conclude that the environment is heavily contaminated with bacteria containing transferable R-plasmids. Man is exposed to the danger of acquiring R-plasmid-bearing coliforms from the environment, and the relative number of R-plasmid-bearing bacteria is increased both by the subtherapeutic use of antibiotics in animal husbandry and the use of antibiotics in human medicine. Antibiotic-resistant bacteria are now so widely distributed in the general environment that it is difficult to relate their appearance to a particular use, but any unnecessary practice that results in the ineffectiveness of antibiotics for the treatment of disease should be eliminated.

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(c) *R-plasmid-bearing human and animal strains of bacteria overlap.* Typing of surface bacterial antigens is used as a means of identifying bacterial strains. Three types of specific surface antigens are associated with the *E. coli* cell: An "O" cell wall lipopolysaccharide antigen, a "K" capsular or envelope antigen, and an "H" flagellar protein antigen which occurs among mobile organisms. The antigens are characteristic of a specific organism, and they serve to identify distinct bacterial types (serotypes) within species. Their presence is detected by the ability of *E. coli* organisms to interact with specific antiserums.

(i) Epidemiological investigations—*E. coli* serotyping: (*a*) Despite the widespread occurrence of R-plasmids in the environment, some workers (Bettelheim et al., Ref. 1) suggested that human *E. coli* and animal *E. coli* were distinct. These workers argued that there were marked differences in serotype distribution in strains isolated from man and animals; they also suggested than animal strains of *E. coli* were not reaching the human population or were failing to implant in the bowel. More recently, however, this same group, Bettelheim et al. (Ref. 2), compared the serotypes of 13,139 strains of *E. coli* isolated from humans with the serotypes of 1,076 animal strains of *E. coli;* 708 different O/H serotype combinations were found. Of these, 520 were found in human strains only, 130 from animal strains only, and 58 O/H serotypes from humans and animals. The authors concluded:

> At first glance the results described in this paper would indeed support the view that human and animal strains of *E. coli* are largely distinct. Second thoughts, however, suggest a little caution in accepting the opinion too firmly.
>
> However thoroughly human or animal stools are examined, only a minute fraction of the total bacterial content is examined, and inevitably strains recorded as being isolated tend to be those that predominate. It is always probable that if examination is continued, further strains may be isolated but after an amount of work that is impracticable in any ordinary investigation. If this is so, it is possible that many of the strains recorded as coming from humans only or from animals only might, with more diligent examination, be recorded as present in both man and animals.

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(*b*) Linton, Howe, Richmond, and their collaborators (Refs. 1 through 4) also conducted extensive epidemiological investigations. They found a wide range of resistant and sensitive O-serotypes of *E. coli* in calves, pigs, and poultry, and they compared these serotypes with those found in the human intestine. The authors found that many O-serotypes common to man were also common to one or more of the three animal species examined. Thus, they concluded that it is impossible to make a clear distinction between "animal" and "human" intestinal strains of antibiotic-resistant *E. coli* based on O-serotyping alone. More importantly, the studies suggest a considerable overlap in the distribution of R-plasmid-bearing O-serotypes in man and in animals. Moreover, the same resistant serotypes, which predominate in the *E. coli* populations from healthy human and animal fecal sources, were also prevalent among R-plasmid-bearing strains from clinical material (Ref. 5).

Because the use of O-serotyping alone as an epidemiological tool has been criticized on the grounds that it is incomplete and inadequate, Howe and Linton (Ref. 2) examined *E. coli* for the K and H antigens as well as the O antigen. They studied 90 strains, 17 chosen at random from human urinary tract infections, 17 from human feces, and 56 from calf feces, all belonging to O-types 8, 9, and 101. The authors found the same K and H antigens in certain strains of the same O-types from each of the three *E. coli* sources. Additionally, K and H antigens associated with these O-serotypes were not specific to antigens associated with these O-serotypes were not specific to *E. coli* isolated from humans or from calves. Although further subdivision of the three O-serotypes was possible by this means, the authors concluded that O-serotyping alone provided a very useful means of distinguishing strains of *E. coli* in a general survey.

These studies show that a similar range of drug-resistant R-plasmid-bearing O-serotypes of *E. coli* have been found in man and the various animal species examined. Furthermore, the studies show that the ratio of drug-resistant to drug-sensitive isolates was much higher in animals than in man (Ref. 2 and 6). Thus the abundance and diversity of drug-resistant R-plasmid-bearing O-serotypes in animals are much greater than that currently found in man, and the serotypes overlap.

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(ii) Direct ingestion evidence: Direct ingestion experiments have also been conducted to show that R-plasmid-bearing *E. coli* of farm origin can colonize the human intestinal tract. In 1969, Smith (Ref. 1) concluded that animal *E. coli* strains were poorer at colonizing the intestine of man than were human *E. coli* strains. However, his observations were based on a single volunteer (himself) and a small number of *E. Coli* strains. Cooke in 1972 (Ref. 2), on the other hand, reported that it was relatively easy to produce temporary colonization of the intestine by *E. coli* strains from both human and animal sources. She reported the persistence of an *E. coli* infection of animal origin in a human volunteer for 120 days following the ingestion of a very large dose.

Other experimental studies (Refs. 3 and 4) confirm that temporary colonization occurs provided a large dose of the organisms is taken, but there is a great deal of biological variation between colonization for different strains and for different human individuals. In normal individuals the carriage of intestinal *E. coli* seems to follow a characteristic pattern. Each person carries one or two resident strains that establish themselves and multiply for months or years. In addition, four or more transient strains are present for a few days or weeks. Strains disappear and are replaced by others. Sometimes, under antibiotic pressure, a new strain suddenly takes over, later disappearing. Strains of *E. coli* thus differ in their ability to colonize man. Although some strains are not well adapted to colonize man, others are able to live in human as well as in animal intestines. The greater the diversity of R-plasmid-bearing O-serotypes that reach the consumer, the greater the probability that one more of these antibiotic-resistant strains will be capable of colonizing man.

Recently, Linton, Howe, Bennet, et al. (Ref. 5) demonstrated that antibiotic-resistant *E. coli* found on a commercially prepared chicken carcass colonized the intestinal tract of a human volunteer. Two strains present on the chicken carcass handled and eaten by the human volunteer were subsequently excreted by her. Both strains were undetectable in the human before contact with the chicken carcass. The strains were shown to be identical in chicken and man by comparing their serotypes (O, K, and H antigens) and R-plasmids. The plasmid complements were determined to be identical by electron microscopy and restriction endonuclease patterns. Restriction endonucleases are enzymes that cleave DNA at specific sites. Physiochemical techniques then visualize these plasmid fragments. The identity of these plasmids can be determined by a comparison of the DNA fragments generated using restriction enzymes with different recognition sequences. The Linton study also suggested that the handling of the uncooked carcass provided a greater opportunity for transmission than does eating cooked meat. The strains persisted for 10 days and the process occurred without feeding any antibiotics to the volunteer during the study. This is consistent with reports of Salmonella infections from animal sources.

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(iii) In vivo studies show that R-plasmids transfer from *E. coli* to pathogens: The ingestion of R-plasmid-containing bacteria can result in in vivo R-plasmid transfer to the normal intestinal flora. When this occurs, the *E. coli* constitute a reservoir of organisms capable of transferring R-plasmids to intestinal pathogens, e.g., Salmonella. The in vivo transfer of R-plasmids has been demonstrated in sheep, mice, calves, pigs, chickens, turkeys, and in the human alimentary tract (Refs. 1 through 8). Generally, in vivo transfer is not as readily detectable as in vitro transfer. In the absence of drug selection, the rate of in vivo R-factor transfer is generally low, and large numbers of resistant donors may be required for transfer (Refs. 1 and 6). Demonstrations of in vivo transfer have usually been achieved by first modifying the normal flora of the alimentary tract by feeding antibiotics, by starvation, or by using germ-free mice or newly hatched chicks, and these procedures probably counteract the inhibitory effects of bile salts, fatty acids, acid pH, and anaerobic conditions of the normal intestinal tract.

These experimental results may not be a true indication of the extent of R-plasmid transfer in natural populations since they often involve individuals who are exposed to restricted numbers and types of donor and recipient organisms. In some instances the methods were not suitable for the detection of low level transfer. However, Smith and Tucker (Ref. 9) studied the effect of antibiotic therapy on the fecal excretion of Salmonella by experimentally infected chickens. The authors found that R-plasmid resistance developed in the indigenous *E. coli* and that very similar resistance patterns then developed in the Salmonella. These results were duplicated in some of the studies submitted by the Animal Health Institute, which are also discussed in depth under Part IV. B. below.

Regardless of the frequency with which R-plasmid transfer occurs in the absence of modifying influences, it has occurred and given rise to antibiotic resistance in bacteria, including pathogens. The conditions of the Smith and Tucker studies mimic those brought about by the practice of feeding subtherapeutic levels of penicillin and other antibiotics to animals. That practice leads to an increase in and selection for R-plasmid-bearing organisms, and it therefore increases the probability of in vivo R-plasmid transfer to pathogens.

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(iv) R-plasmid compatibility studies: Another FDA study (Ref. 1) examined the compatibility properties of more than 100 R-plasmids from *E. coli* and *Salmonella* isolated from animals in or-

der to determine whether the plasmids are related to those isolated from man. The usual method of genetically classifying plasmids is based on their ability to exist with each other in the same bacterium. Genetically unrelated plasmids can exist in the same host, and they are called compatible. On the other hand, related plasmids cannot coexist, and they are called incompatible. Plasmids belonging to the same incompatability group are presumed to be related.

The FDA study showed that the R-plasmid incompatibility groups seen in animal isolates show the same distribution as those found in human isolates. This suggests that human and animal bacterial populations contain the same plasmids.

A more direct apporach for examining the relationships between plasmids is to measure the proportion of DNA sequences (that is, the number of similar or identical genes) that are common to any two plasmids (DNA–DNA hybridization). R-plasmids belonging to the same incompatibility groups of human and animal origin are identical when examined by DNA–DNA hybridization techniques (Refs. 2 and 3). Restriction endonuclease activity has also confirmed the similarity of R-plasmids isolated from enteric organisms of human and animal sources (Ref 4). Therefore, the Director must conclude that R-plasmids of human origin are indistinguishable from those of animal origin.

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(v) Hazards: Although antibiotic-resistant *E. coli* in the intestinal tract of humans may generally cause no immediate problems to an individual, under proper circumstances their presence may lead to dangerous situations. For example, *E. coli* is the most common cause of urinary tract infections in man and commonly arises from a person's own intestinal flora. While sulfonamides are generally the drug of choice, a significant number of infections with sulfonamide-resistant strains are now reported, necessitating treatment with penicillin.

Resistant *E. coli* in the intestine of man also constitute a reservoir of organisms capable of transferring R-plasmids to intestinal pathogens. Perhaps the greatest hazard to human health arising from the use and misuse of antibiotics is the large reservoir of plasmid-mediated resistance genes in the normal flora of animals and man and present in the environment—resistance that can be transferred from nonpathogenic to pathogenic organisms.

In recent years the emergence of R-plasmid-mediated resistance in dangerous pathogens has been identified in epidemics around the world. A strain of *Salmonella typhi* carrying an R-plasmid-mediating resistance to chloramphenicol caused an epidemic of typhoid fever in Mexico. Transferable chloramphenicol resistance has also become common in *S. typhi* isolated in India, Vietnam, and Thailand (Ref. 1). The recent epidemic of drug-resistant *Shigella dysenteria* infection in Central America (Ref. 2) is another example of an epidemic disease that was no longer susceptible to treatment by the antibiotics that had previously been used for its treatment. Plasmid-mediated resistance has been reported in strains of *Bordetella bronchiseptica* (Ref. 3), and FDA scientists have demonstrated plasmid-mediated resistance to penicillin, tetracycline, streptomycin, and sulfonamide in strains of *Pasteurella multocida* and *P. haemolytica*, both of which cause serious diseases in animals (Refs. 3 and 4).

Recent studies (Refs. 5 through 12) have also shown that the genes specifying resistance to ampicillin, tetracycline, kanamycin, chloramphenicol, trimethoprim, and streptomycin reside on DNA sequences that are able to translate or move from plasmid to plasmid as a discrete unit, or from a plasmid to the bacterial chromosome. Therefore, in addition to movement of resistant bacteria from animals to man and the transfer of R-plasmids between bacteria, the genes that reside on the plasmids can themselves migrate from plasmid to plasmid by translocation. Furthermore, an R-plasmid does not have to be maintained stably within a cell to donate its resistant genes to a recipient chromosome or an indigenous plasmid.

Most bacterial species possess indigenous plasmid gene pools. In fact, plasmids have been found in all species of bacteria examined. The function of these plasmids is often unknown, but they could serve as effective recipients for the insertion of translocatable genes. The recent emergence of ampicillin-resistant strains of *Haemophilus influenzae* and penicillin-resistant strains of *Neisseria gonorrheae* represent alarming examples of the extension of the R-plasmid gene pool (Refs. 13 and 14). The resistance genes found in both species are identical to those previously found only in *E. coli* and other enteric organisms.

The World Health Organization prophetically warned (Ref. 15):

> The point will ultimately be reached at which the transfer of resistance to pathogens becomes inevitable and the larger the pool, the greater is this possibility. Moreover, the wide the distribution of R+ (R-factor) enterobacteria the greater the possibility that R-plasmids may emerge that can cross biological barriers so that they can perhaps enter bacterial species and genera apparently widely different from their original enterobacterial hosts.

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4. *Director's conclusions.* The holders of the approved NADA's for subtherapeutic penicillin-containing products were required to show that the subtherapeutic use of penicillin does not increase drug resistance (increase the pool of R-plasmid-bearing) organisms in animals. If they were unable to show that subtherapeutic penicillin use does not increase the pool of R-plasmid-bearing organisms in animals, the holders were then required to show that the R-plas-

mids are not transferable from animals to man. They failed to do any of this.

The evidence shows that the pool of R-plasmid-bearing organisms, particularly in *E. coli*, is increasing, and that the increase is due at least in part to the subtherapeutic use of penicillin in animal feed. Further evidence shows that *E. coli* contribute their R-plasmids to man through his direct contact with animals, through his direct contact with *E. coli*-contaminated food, and by widespread presence of the R-plasmids in bacteria in the environment. Studies also show that there is no strict distinction between the *E. coli* that colonize animals and those that infect man. On the contrary, there is considerable overlap in these strains, and there is also an overlap in the enteric bacterial R-plasmid population in humans and animals. This evidence is derived from epidemiology studies, bacterial ingestion studies, and compatibility studies of the normal intestinal flora of man and animals. These bacteria may donate their R-plasmid to pathogens in man and animals even when transient, and the NADA holders have submitted no evidence on the degree of colonization, if any, that is necessary for this transfer to occur. Accordingly, the Director concludes that the holders of the approvals for the subtherapeutic penicillin-containing products for use in animal feeds have failed to satisfy the requirements of § 558.15 and criterion 1 of this notice.

B. Shedding and Resistance Characteristics of Salmonella (Criterion 2)

1. *Background.* A second area of concern, related to the increase in the pool of R-plasmid-bearing bacteria, is the possibility that the subtherapeutic use of antibiotics in animal feeds may lead to an increase in the duration or quantity of live *Salmonella* excreted by the animal receiving the drug(s), which will increase contamination of the environment with pathogens. This concern was generated in part by reports that antibiotic therapy in human salmonellosis patients had resulted in prolonged *Salmonella* shedding and favored the acquisition of resistence in *Salmonella*.

Aserkoff and Bennett (Ref. below), for example, presented data on the effect of antibiotic therapy on the excretion of *Salmonella* in the feces of human infected with acute salmonellosis. Following a large *S. typhimurium* epidemic caused by eating contaminated chicken, feces of untreated patients and patients treated with tetracycline, ampicillin, and chloramphenicol were examined for *Salmonella*, and the antibiotic susceptibility of the *S. typhimurium* strains was determined. Patients generally received the recommended regimen of antibiotic therapy (1 gram per day). Fecal samples from 87 patients not receiving medication and 185 patients treated with antibiotics were examined. Of the patients treated with antibiotics, 65 percent were shedding *Salmonella* 12 days after infection, and 27 percent were positive 31 days after infection. In the untreated patients, however, *Salmonella* shedding was observed in 42.5 percent at day 12 and 11.5 percent at day 31.

Antibiotic therapy also favored the acquisition of drug resistance by the infecting strain of *Salmonella*, which was initially susceptible to antibiotics. Of the patients receiving antibotics, 10 excreted resistant *Salmonella*, while none of the 87 untreated patients excreted resistant *Salmonella* ($P<.05$). The antibiotic resistance acquired in the *Salmonella* strain was shown to be transferable. Because antibiotic treatment increased shedding in human salmonellosis, FDA became concerned that subtherapeutic antibiotic, penecillin) administration in animal feeds would prolong *Salmonella* shedding in animals, and for this reason the agency established criterion 2.

REFERENCE

Aserkoff, B., and J. V. Bennett, "(Effect of Antibiotic Therapy in Acute Salmonellosis on the Fecal Excretion of Salmonella," New England Journal of Medicine, 281:636-640, 1969.

2. *Criterion*—(a) *Shedding.* Controlled studies were to be designed to determine whether the administration of an antibacterial drug at subtherapeutic levels would result in an increase in the relative quantity, prevalence, or duration of shedding of *Salmonella* that are pathogens in animals. *Salmonella* are often found in the intestinal tract of man and animals, and the small intestine and colon are the primary sites of multiplication. After penetrating the epithelial lining, they multiply and elicit an inflammatory response. Most *Salmonella* infections are limited to the gastrointestinal tract, producing the clinical symptom termed "gastroenteritis." One of the more common strains, *Salmonella typhimurium*, causes diseases in both man and animals.

When an animal is infected with these bacteria, the live organisms are excreted in the feces ("shedding"). The quantity of *Salmonella* in the feces can be determined by a bacteriological procedure termed a "standard plate count." A specific amount of fecal material is diluted and spread on a semisolid bacterial growth medium which is selective for the growth of *Salmonella*. After a sufficient time for growth, individual colonies are counted and recorded as the number of *Salmonella* per gram of wet feces. The proportion of antibiotic resistant *Salmonella* in fecal specimens is independent of the quantity of Salmonella shed.

(b) *Resistance characteristics.* Controlled studies were to bedesigned to determine whether the administration of penicillin at subtherapeutic levels would result in an increase in the proportion of antibiotic resistant *Salmonella*. *Salmonella* isolated from feces can be tested for their susceptibility to various antibiotic drugs. *Escherichia coli*, a normal component of the intestinal flora, were also to be examined to determine their resistance spectrum sinffice oral administration of certain antibiotics, whether at therapeutic or subtherapeutic levels, has been shown to result in an increased proportion of indigenous *E. coli* that contains R-plasmids. These *E. coli* can serve as a reservoir of R-plasmids that can be transferable to other *E. coli* or to *Salmonella*.

3. *AHI Studies on the Effects of Subtherapeutic Levels of Penicillin in Animal Feed in Chickens.* On behalf of the NADA holders, the Animal Health Institute submitted the following study to address the criterion.

(a) *Experimental design.* The Animal Health Institute submitted an experiment in which the effects of subtherapeutic levels of procaine penicillin (with or without streptomycin) in feed were investigated. The duration, quantity and antibiotic susceptibility of a *Salmonella* strain inoculated into chickens were compared in medicated and nonmedicated chickens.

Also, FDA specified that prestudy (baseline) *E. coli* antibiotic resistance levels should be under 20 percent. This value was thought to provide a reasonable level for detecting any change in the amount of antibiotic resistance resulting from administration of subtherapeutic antibiotic levels since, if the initial R-plasmid level is too high, a small change in resistance is difficult to detect.

While others served as environmental controls, 1-day-old chicks were divided into six groups, artificially infected with *Salmonella*. Each group received medicated or nonmedicated diet, according to the following plan:

Room	Group	Inoculation of salmonella 1.6×10^{9}	Antibiotics and levels used in feed	Number of chickens in experimental group
1	A	Yes	None	10
2	B[1]	Yes	Procaine penicillin 50 g/ton	10
	B[2]	Yes	Procaine penicillin 12.5 g/ton, streptomycin 37.5 g/ton	10
3	C	No[1]	None	5
	D[1]	No[1]	Procaine penicillin 50 g/ton	5
	D[2]	No[1]	Procaine penicillin 12.5 g/ton, streptomycin 37.5 g/ton	5

[1] Environmental controls.

Groups A and B were used to determine the influence of penicillin or penicillin-streptomycin on shedding after experimental infection and the development of drug resistance by *Salmonella* and *E. coli*, with group A serving as a nonmedicated control group. Groups C and D were controls used to monitor the environment, and the effects of the drugs in the absence of experimental infection. To assure the absence of naturally occurring Salmonella prior to the study, the sponsors examined prestudy fecal samples. The samples were grown in a selective media, brilliant green agar, and serotyping was also done. By

this procedure, the birds were determined to be negative for Salmonella before the experiment began, and any bacteria suspected were further tested biochemically and serologically.

The infecting *Salmonella* (*S. typhimurium* 289–1, a poultry strain, chromosomally resistant to nalidixic acid and sulfonamides) was given by oral gavage. Fecal specimens from each chicken were diluted in phosphate buffered saline and appropriate dilutions were spread on growth medium selective for the nalidixic acid-resistant *S. typhimurium* used to infect the birds. The number of Salmonella growing on the medium was recorded as the number of *S. typhimurium* per gram of wet feces.

Presumptive *E. coli* isolates were obtained from EMB plates inoculated with diluted fecal material. The antibiotic resistance spectrum for *E. coli* isolates was also measured in accordance with the Standardized Disc Susceptibility Test set forth in § 460.1(c) (2) (21 CFR 460.1(c) (2)) for ampicillin, tetracycline, chloramphenicol, kanamycin, nitrofurantoin, streptomycin, sulfathiazole, and triple sulfa. The *E. coli* isolates were tested only twice prior to infections and once at the termination of the study (28 days), while the Salmonella isolates were tested nine times during the study.

Salmonella isolates were selected from the selective medium, brilliant green agar plates containing nalidixic acid, and were serotyped. Antibacterial susceptibility tests for ampicillin, tetracycline, chloramphenicol, kanamycin, nitrofurantoin, streptomycin, sulfathiazole, and triple sulfa were carried out in accordance with the Standardized Disc Susceptibility Test in § 460.1(c) (2). The isolates were tested on days 2, 4, 6, 8, 10, 13, 14, 21, and 28 of the experiment.

Clinical records were maintained on body weights, disease symptoms, mortality, and gross and microscopic pathology.

(b) *AHI's summary of the results.* (i) Shedding: Initially, on day 2, group B_1 (penicillin 50 grams/ton) shed a geometric mean number of 10^6 Salmonella per gram of feces; and during the remainder of the study, the geometric mean shed by the group decreased steadily. At the end of the study, the number shed was below the reliable limit of quantitation, less than 10^2 organisms per gram of feces. Group A, the nonmedicated control group, on the other hand, shed 10^7 organisms on day 2, and continued to shed a greater number of organisms than the treatment group ($P < .05$) throughout the remainder of the study. None of the environmental control groups, groups C and $D_{1,2}$ shed detectable amounts of Salmonella.

The prevalence of *S. typhimurium* was estimated by comparing the fraction of Salmonella positive fecal samples in the penicillin treatment group (group B_1) to that for the nonmedicated control group (group A) from all samplings. Thus, 69 out of 90 specimens (77 percent) examined from nonmedicated (control) animals were positive for *S. typhimurium*, while only 36 of 81 specimens (41 percent) in the penicillin treatment group were positive for *S. typhimurium*. The results represent statistically significant differences ($P < .01$) between the incidence of Salmonella positive samples in the treatment group and in the nonmedicated control group.

Duration of shedding was measured by determining the length of time that fecal samples were positive for Salmonella, or analyzing the time required for quantities of Salmonella shed to reach a common value. At least three nonmedicated birds shed Salmonella in their feces throughout the experiment, and four were positive 28 days after infection. In contrast, by day 12, only one bird receiving penicillin was positive, and none were positive on day 28. The length of time positive counts persisted was significantly longer ($P = .05$) in nonmedicated controls than for the penicillin-treated group.

Liver, spleen, and cecal tissues from all animals were necropsied, and samples tested for Salmonella. All tissues were negative.

The AHI concluded that feeding a diet containing a subtherapeutic level (50 grams/ton) of penicillin to chickens that were experimentally infected with *S. typhimurim* did not increase the quantity, shedding, or prevalence of Salmonella in fecal specimens, nor did it increase the quantity of Salmonella isolated from liver, spleen, or cecal tissue. In the opinion of the AHI, the evidence from this study suggests that subtherapeutic use of pencillin in chickens reduced the quantity, shedding, and prevalence of Salmonella.

(ii) Resistance characteristics: (*a*) *E. coli.* According to the two pretreatment samples, the proportion of *E. coli* isolates that were drug resistant was low (below 6 percent), except for resistance to sulfonamides which was greater than 85 percent. But at the experiment's end, AHI found that the resistance to ampicillin, chloramphenicol, kanamycin, and nitrofurantoin was significantly higher ($P < .01$) in the penicillin environmental control groups (D_1) than the control birds (C). Ampicillin resistance also significantly increased in the infected birds that received penicillin. Resistance to sulfonamides remained at the pretreatment level of greater than 85 percent, although the figure in the environmental control groups decreased.

(*b*) *Salmonella.* Prior to inoculating the birds, the infecting strain of *S. typhimurium* was resistant only to sulfonamides and nalidixic acid, the nontransferable marker. *S. typhimurium* strains showed a significant increase in ampicillin resistance on days 12 ($P < .01$) and 14 ($P < .05$). No other significant increases were observed for the other antimicrobials in the test.

The AHI then concluded that the penicillin supplemented diets significantly increased the percentage of *E. coli* that were resistant to ampicillin. In the Salmonella, the AHI found no significant difference in drug-resistant isolates when all the chickens in the trial were considered. But among the animals shedding Salmonella, i.e., the medicated groups, the nonmedicated control, the birds exposed to subtherapeutic antibiotic pressure (both penicillin and penicillin-streptomycin), a significantly greater proportion shed Salmonella that were resistant to ampicillin than in the nonmedicated groups.

(c) *The Director's analysis.* (i) Shedding: (*a*) The Director does not disagree with some conclusions drawn by AHI about this study. Feeding a subtherapeutic level of penicillin did not apparently increase the quantity of Salmonella shed in fecal material; it did not appear to increase the number of Salmonella in liver, spleen, and cecal tissue; and it did not increase the number of positive chicken tissues.

The Director, however, disagrees with the conclusion of AHI that feeding penicillin at 50 grams/ton did not increase the duration or prevalence of Salmonella shedding because the procedures that were used to determine these paramotors were inadequate. The information necessary to determine Salmonella duration and prevalence is whether Salmonella are present in the feces, not the quantity of Salmonella in the feces. After the animals were infected with Salmonella in this experiment, fecal specimens were processed by diluting them and then plating on the surface of agar plates. Clones growing on the plates were subsequently counted to provide information on number of Salmonella per gram of feces. As the study progressed, however, the number of Salmonella shed decreased in both groups, and this procedure is inadequately sensitive to detect small numbers of Salmonella. Good microbiological practice requires the use of an enrichment procedure for culturing. An enrichment procedure involves the incubation of a fecal sample in a selective broth to increase the number of Salmonella before plating on the agar. This increases the likelihood that Salmonella will be detected because other genera are being simultaneously inhibited. The enrichment procedure is recommended for examination of fecal specimens where small numbers of Salmonella may be present, as in the case of subjects in the carrier state. In its section about processing of specimens from the bacterial family, Enterobacteriaceae (Salmonella is a member of this family), the "Manual of Clinical Microbiology," 2d edition, American Society for Microbiology, Washington, D.C., p. 194 (1974) is clear:

> It always is advisable to employ enrichment media in the examination of various kinds of specimens, and their use is practically essential when dealing with fecal specimens from carriers of suspected carriers.

In an FDA experiment, the agency studiesd Salmonella shedding by swine (Ref. below). Through careful study, 28 percent more samples (136 rather than 94 from the 151 examined) were determined to be Salmonella positive when an enrichment procedure was used. In another similar study by FDA, 95 percent rather than 60 percent of 242 samples were found Salmonella positive by media enrichment. Enrichment procedures had been requested by FDA

during the protocol development stage; thus, the AHI determination of prevalence and duration for this study was considered inadequate.

REFERENCE

Rollins, L. D., FDA Project 108.

(b) The shedding study was conducted in three rooms. The chickens that were experimentally infected with *S. typhimurium* were maintained in two separate rooms, and the third room housed the noninfected environmental control animals. In one of the rooms containing infected birds, the chickens received only nonmedicated feed. However, all birds that were infected with Salmonella and receiving medication were placed in the same room. These birds received one of three different medicated diets, either penicillin, penicillin plus streptomycin, or sulfaquinoxaline. Although the chickens were maintained in separate cages within the same room, no birds were placed in this room to determine if bacteria from one study group were flowing to another study group within the room (environmental control). The rise in levels of resistance to antibiotics in noninfected, nonmedicated group A, as well as in the antibiotic-treated groups B_1 and B_2, suggests that some cross-contamination might have occurred or that contamination from the environment might have occurred. Such contamination of control groups makes it more difficult to detect differences in the increase of drug resistance between the experimental and control animals.

An FDA-sponsored contract (71–269) showed the relative ease by which cross-contamination occurs between various study groups. These groups were under similar or more adequate isolation conditions than the chickens in the AHI study.

Nevertheless, analysis of drug resistance data obtained from bacteria isolated from the various groups maintained in Room 2 of the AHI study indicates there are differences in drug resistance between groups. This suggests that when R-plasmids are present, regardless of their source, they may be transferred even in the absence of antibiotic pressure.

(c) When the shedding studies were initially requested, the optimum duration of such studies was unknown, although the 28-day duration appeared adequate. Data later generated under FDA sponsorship (contract 71–269) show that shedding patterns change after 30 to 50 days, longer than the length of the 28-day AHI experiment. Some studies have shown Salmonella shedding to be decreasing in both medicated and nonmedicated groups early in the experiment, with the shedding initially decreasing faster in the medicated group. In several of these experiments, approximately 55 days after initiating the experiment, the Salmonella shedding patterns reversed and shedding in the medicated birds increased, while shedding in the nonmedicated birds remained constant or continued to decrease. In the Director's opinion, the phenomenon is easily explained. Initially, the antibiotic attacks sensitive organisms and as these predominate, little shedding is observed. But, as the antibiotic-resistant organisms remain and become dominant in the population, shedding increases.

(d) The 28-day duration of the chicken studies should also be considered in relation to the life of a commercial broiler chicken, usually about 7 to 8 weeks. Although some changes in shedding pattern occurred beyond 6 weeks, in normal commercial production, groups of broilers are raised continuously with one group immediately following another. The production facilities may be cleaned between groups; however, the facilities are not sterilized. Bacteria left from a preceding group of birds are available to infect the birds that follow, and some of the microbiological changes that occur may be perpetuated in subsequent birds. Thus, if an antibiotic is used in the feed of each group of birds, it would have an opportunity to act over a long period of time. For these reasons, the Director now believes it is necessary to use an experimental design that allows sufficient evaluation of the effect of time of antibiotic usage on shedding.

(ii) Resistance characteristics: (a) *E. coli.* A major concern about occurrence of drug resistance in *E. coli* that are indigenous to the digestive tract is their potential for donating drug resistance to pathogens such as Salmonella. The Director agrees with the AHI analysis that feeding chickens the penicillin supplemented diet significantly increased ($P<.05$) the number of *E. coli* isolates that were resistant to ampicillin. But other aspects of the drug resistance characteristics of *E. coli* are also critical to an appropriate analysis of the data. Although the proportion of *E. coli* resistant to sulfonamides was high in all the groups before treatment and before inoculating the chickens with Salmonella, the bacteria were relatively susceptible to the other antibiotics tested. Results from one sample collected from each bird after penicillin treatment and inoculation with *S. typhimurium*, however, indicate that the proportion of *E. coli* resistant to streptomycin and tetracycline increased in all groups—environmental controls, nonmedicated controls, and treatment groups. This suggests bacteria that were resistant to tetracycline, streptomycin, and perhaps sulfonamides colonized the animals in the experimental facility.

(b) *Salmonella.* Although the total quantity of Salmonella shed decreased, the percentage of drug-resistant Salmonella shed increased, which is crucial. For birds that were shedding Salmonella, feeding penicillin resulted in a significantly greater proportion of Salmonella resistant to ampicillin ($P<.05$), which is consistent with the AHI analysis. The Director agrees with AHI that feeding subtherapeutic penicillin resulted in a significant increase in both the proportion of ampicillin-resistant *E. coli* and Salmonella.

For all of the foregoing reasons, the Director concludes that the study has failed to prove that the subtherapeutic use of penicillin in chickens satisfies the criterion and has failed to show that such use is safe.

4. *AHI Studies on the Effects of Subtherapeutic Penicillin in Animal Feed in Swine*—(a) *Experimental design.* To measure Salmonella shedding in swine and the transfer of drug resistance to Salmonella, AHI submitted a study that was similar in design to the previously described chicken study. This study was also subject to the same experimental conditions that FDA imposed on the chicken study, i.e., the base line incidence of resistance to drugs used in human clinical medicine in the indigenous flora of the test animals was not to exceed 20 percent.

Swine were divided into six groups, three of which were infected with Strain No. 58 DO 13C *Salmonella typhimurium* (swine) characterized as sulfonamide resistant. One noninfected and one infected group received diets containing either no medication, procaine penicillin, or procaine penicillin plus streptomycin according to the following design:

Room number	Group	Antibiotic and level used in feed	Inoculation of salmonella (1.3×10^{11} dose)	Number of pigs in experimental group
1	A	None	Yes	10
2	B_1	Procaine penicillin (50 g/ton)	Yes	10
	B_2	Procaine penicillin (12.5 g/ton), streptomycin (37.5 g/ton)	Yes	10
3	C	None	No	5
	D_1	Procaine penicillin (50 g/ton)	No	5
	D_2	Procaine penicillin (12.5 g/ton), streptomycin (37.5 g/ton)	No	5

(i) Shedding: Groups B_1 and B_2 were used to test the influence of penicillin on shedding and resistance of Salmonella in the test animals, with group A serving as a nonmedicated control group. Groups C, D_1 and D_2 were used as environmental controls to monitor whether swine administered the drug but not inoculated remained Salmonella free.

Orally via the diet, 6-week-old pigs were experimentally infected with an inoculation of 1.3×10^{11} Salmonella, 5 days after beginning their test diet. Preinfection fecal specimens were free of naturally occurring Salmonella for all test animals. For all pigs in each group, fecal samples were taken on days 2, 4, 6, 8, 10, 12, 14, 21, and 28 postinfection to quantitate the Salmonella. One-gram samples of fecal specimens from each test animal were diluted in phosphate-saline solution and plated in duplicate on brilliant green agar containing 0 and 20 micrograms/milliliter of streptomycin. After incubation, characteristic clones of Salmonella were recorded as total counts/

gram of wet feces. All pigs were killed and necropsied 28 days after the infection.

One-gram samples of aseptically collected liver, spleen, ileocecal lymph node, and cecum were minced and incubated in tetrathionate brillant green broth, and subsequently plated on brilliant green agar to determine the presence of Salmonella. Clinical records were maintained on body weights, mortality, and gross and microscopic pathology.

(ii) Resistance characteristics: (*a*) *E. coli.* Coliform counts were obtained from EMB plates inoculated with homogenized fecal samples. One gram of each sample was plated in duplicate on EMB agar containing 0 and 20 milligrams/milliliter of streptomycin. Antibiotic susceptibility tests were conducted on clones obtained from two prestudy samples and one poststudy sample from each animal in accordance with the Standardized Disc Susceptibility Tests in § 460.1(c) (2). Five cones from each specimen were selected from the streptomycin plates and were tested for susceptibility to ampicillin, tetracycline, chloramphenical, streptomycin, kanamycin sulfate, nitrofurantoin, and sulfathiazole.

(*b*) *Salmonella.* Five clones of *Salmonella* selected from the brilliant green fecal count plates were tested for antibacterial susceptibility to ampicillin, tetracycline, chloramphenicol, streptomycin, kanamycin sulfate, and nitrofurantoin, sulfathiazole, and triple sulfa, in accordance with the Standardized Disc Susceptibility Test in § 460.1(c) (2). When there were less than five clones of *Salmonella,* the number of clones picked corresponded to the actual number present on the plates.

(b) *AHI's summary of the results.* (i) *Shedding:* AHI reported that the number of *Salmonella* receovered per gram of wet feces diminished with time in all groups, and the number of organisms recovered from the medicated groups after day 2 was consistently less than the numbers recovered from the nonmedicated control group. These numbers represent average counts of clones growing on agar that did not contain streptomycin since no *Salmonella* grew on plates containing streptomycin. No *Salmonella* were isolated throughout the experiment from any of the environmental control animal (Groups C, D_1, and D_2). From this the AHI concluded that the presence of antibacterials in animal feeds reduces the quality and persistence of *S. typhimurium* in experimentally infected pigs.

(ii) Resistance characteristics: (*a*) *E. coli.* AHI concluded that penicillin supplemented diets significantly increased ($P<.01$) the number of *E. coli.* resistant to chloramphenicol. Similarly, penicillin/streptomycin supplemented diets significantly increased ($P<.05$) the number of *E. coli.* resistant to streptomycin.

(*b*) *Salmonella.* When the experimentally infected pigs in the medicated groups were compared to the nonmedicated control group, AHI concluded that feeding penicillin or penicillin/streptomycin at subtherapeutic levels did not increase the percent of pigs carrying resistant *Salmonella.* It also concluded that there were no significant differences in the percentage of resistant clones isolated from pigs in the penicillin group and the control group when all the pigs were considered (nonmedicated controls, environmental controls, and treatment groups).

(c) *Director's analysis.* (i) Shedding: The Director again does not totally disagree with AHI's conclusions concerning *Salmonella* shedding in swine. He agrees that, in this case, feeding a subtherapeutic level of penicillin apparently neither increased the quantity of *Salmonella* being shed in the pig's fecal material, nor increased the number of *Salmonella* in liver, spleen, ileocecal lymph node and cecum. Feeding penicillin also did not increase the number of swine tissues (liver, spleen, ileocecal lymph node and cecum) that were positive for *Salmonella.* However, the Director disagrees with the AHI conclusion that feeding swine penicillin at 50 grams/ton did not increase the duration or prevalence of *Salmonella* sheeding, because the procedures that were used to determine these parameters were inadequate. The information necessary to determine duration and prevalence of *Salmonella* shedding is whether feces contain any *Salmonella,* even in very low numbers, rather than the quantity of *Salmonella* present in the feces, which AHI measured. After the animals were infected with Salmonella, fecal specimens were processed by diluting and then plating the dilutions on the surface of agar plates. Enrichment procedures were not used.

(ii) Resistance characteristics: (*a*) *E. coli.* As in the chicken study, the data available on the occurrence of various drug resistances in *E. coli* are limited; nevertheless, they are sufficient to draw general conclusions. Susceptibility tests from streptomycin-containing plates show a high proportion of multiple-resistant *E. coli* in all groups prior to treatment, i.e., treatment groups, nonmedicated controls, and environmental controls. This is contrary to the recommendations of the FDA guidelines established for these studies. Data from posttreatment plate counts (one for each pig) indicate that the proportion of *E. coli* resistant to streptomycin remained high throughout the experiment and was similar for both the penicillin treatment group (group B_1) and the nonmedicated group (group A). The results are not unexpected because the high initial proportion of drug-resistant organisms makes it difficult to detect differences in the proportion of drug-resistant organisms caused by antibiotic administration.

A more acceptable procedure for determining the proportion of isolates resistant to a particular drug is to select clones from drug-free agar plates for susceptibility testing. A higher proportion of drug-resistant bacteria will be isolated on antibiotic-containing agar than with the random choice of a standard drug susceptibility test using normal agar.

Further, AHI has injected an element of bias in reporting the *E. coli* information. Only the clones that were growing on the streptomycin-containing agar plates were tested for susceptibility to multiple antibiotics. This procedure will reveal the drugs in addition to streptomycin to which the isolate was resistant, but a high proportion of the streptomycin-resistant isolates were also resistant to tetracycline and the sulfonamides.

Selecting clones from streptomycin-containing agar for further susceptibility testing is acceptable for determining what resistances, in addition to streptomycin, may be present. Only those cells resistant to streptomycin, alone or in a pattern with other antibiotics, will grow on agar containing streptomycin. However, cells may be present in the population that are susceptible to streptomycin but are resistant to one or more other drugs. For example, ampicillin-resistant bacteria might be missed. These cells would not grow on the agar containing streptomycin, and the procedures used by the AHI would not report them.

(*b*) *Salmonella. Salmonella* were isolated from both the nonmedicated control group (group A) and the penicillin treatment group (group B_1). Isolates that were singly and multiply drug resistant were observed, as well as isolates with resistance to ampicillin, tetracycline, chloramphenicol, nitrofurantoin, kanamycin, and stretomycin. The strain of *Salmonella* used to infect the animals was initially resistant only to sulfonamides when the animals were inoculated. In both the nonmedicated control group and the penicillin treatment group, the proportion of *Salmonella* isolates that were resistant to each drug tested was similar, and a significant proportion of *Salmonella* isolates were resistant to at least one of the following: ampicillin, tetracycline, and streptomycin.

The principal purpose of this experiment was to determine whether feeding of penicillin at subtherapeutic levels results in an increase of drug-resistant *Salmonella.* One way by which *Salmonella* become resistant is by transfer of drug resistance from the indigenous flora, e.g., *E. coli,* of the gut; therefore, the proportion of indigenous organisms in the gut carrying drug resistance directly affects the ability to detect differences due to antibiotic treatment. For this reason the effect that subtherapeutic penicillin has on increasing the proportion of drug-resistant *E. coli* was initially analyzed.

A high porportion of indigenous *E. coli* were drug resistant before treatment, which minimized or negated the observable effect that antibiotic treatment would have on the indigenous gut flora. Since the effect of antibiotic pressure on the indigenous flora was the initial step in the process under study, the study is invalid for demonstrating in a precise manner the effect of feeding subtherapeutic levels of penicillin on occurrence of resistance in *Salmonella.*

An unexplained inconsistency also invalidating the study is the fact that during the study no streptomycin-resistant *Salmonella* grew on the brilliant green agar (BGA) containing streptomycin. However, in subsequent sensitivity testing in the experiment it was determined that many of the *Salmonella* clones isolated at different times on plain BGA were indeed resistant to streptomycin as determined by the standard Kirby-Bauer disc susceptibility test.

A third deficiency undermines the validity of the study. The Director found that 70 to 100 percent of the indigenous *E. coli* in the test swine were resistant to tetracycline, streptomycin, and sulfonamide, and 20 to 50 percent were resistant to ampicillin and kanamycin. He also found that resistance to chloramphenicol and nitrofurantoin had occurred, but to a lesser extent. Nevertheless, in both the medicated animals and nonmedicated animals, the Director found that the resistance paterns corresponded.

Before the study began, the *Salmonella* were resistant only to the sulfonamides. On the basis of the disc susceptibility test, the Director found the following resistance pattern had evolved during the course of the study:

Percent resistant salmonella isolates

Drug	Non-medicated group A	Penicillin treatment group B 1
Streptomycin	53.0	48.0
Tetracycline	43.0	41.0
Ampicillin	69.0	58.0
Kanamycin	12.0	18.0
Chloramphenicol	3.6	5.6
Nitrofurantoin	8.0	3.6
Number of isolates	247.0	195.0

Resistance was transferred to *Salmonella* in the nonmedicated group at a rate at least equal to that of the medicated group. It is thus apparent that *Salmonella* readily became resistant to ampicillin, tetracycline, and streptomycin when exposed to the R-plasmids of *E. coli* present in the gut. This reaffrms the results observed in the chicken study, as well as the studies by Pocurull et al., Neu et al., and Smith and Tucker (Refs. 2, 3, and 6). Once a sufficient number of R-plasmid-bearing bacteria, principally *E. coli,* are present, the *E. coli* donate their R-plasmids in the absence of antibiotic pressure. Accordingly, the Director concludes that the presence and proportion of R-plasmid-bearing donors were responsible for the resistance in *Salmonella.*

Another safety question may be raised by the high *E. coli* resistance found in the swine used in this study; 70 to 100 percent of the *E. coli* were resistant to tetracycline, streptomycin, and sulfonamides, and 20 to 50 percent resistant to kanamycin and ampicillin. Yet, in the Gustafson study cited below (Ref. 7), in typical swine going to slaughter, there were no *E. coli* resistant to ampicillin, although 17 of 31 isolates were multiply resistant to other antibiotics.

For all of the foregoing reasons, the Director concludes that this study has failed to prove conclusively that subtherapeutic penicillin use in swin satisfies the criterion and has thus failed to show that such use is safe.

5. *Questions Raised by Other Studies of Salmonella*—(a) *CDC reports.* The Center for Disease Control (CDC) has maintained a national *Salmonella* surveillance program since 1963. The reported incidence of salmonellosis increased until approximately 1973, when it reached 27,000. The level of reported cases averaged 10.77 per 100,000 population from 1970 through 1974, and true incidence may be far higher because of underreporting. But the reported cases from antibiotic resistant *Salmonella* have continued to increase. *Salmonella typhimurium,* which is the most common *Salmonella* strain in animals, is the resistant strain most often reported in man and animals. More importantly, the number of antibiotic resistant strains of *S. typhimurium* isolated and reported almost doubled between 1967 and 1975, and the increase in antibiotic resistance in other *Salmonella* serotypes almost tripled during that period. Further, in addition to the fact that the number of *Salmonella* strains resistant to 6 or more antibiotics increased almost 10 times, the percentage of multiply resistant strains that are "super resistant" (containing resistance to 6 or more antibiotics) increased almost 7 times (Refs. 1 and 1a).

(b) *FDA survey.* Pocurull, Gaines, and Mercer (Ref. 2), in a 1971 survey, report that *Salmonella* strains isolated from outbreaks of salmonellosis in animals were bearing R-plasmid-mediated resistance to antibiotics. *Salmonella* isolates gathered in diagnostic laboratories of most States from outbreaks of salmonellosis in pigs, cows, chickens, and turkeys were tested for their susceptibility to ampicillin, tetracycline, dihydrostreptomycin, cephalothin, sulfamethoxypyridazine, colistin, chloramphenicol, furazolidone, neomycin, polymyxin, and nalidixic acid. Of the 1,251 strains studied, 75 percent were resistant to one or more antibacterial drugs, 40 percent were resistant to two or more antibacterials, and 21 percent were resistant to three or more antibacterials. But an even higher incidence of multiply resistant cultures was observed in *S. typhimurium,* which was again the most commonly isolated pathogen.

(c) *Neu, Cherubin, Longo, Flouton, and Winter studies.* Recently, Neu et al. (Ref. 3) examined the antimicrobial susceptibility of 718 *Salmonella* isolates from humans and 681 from animals. They compared the current prevalence of antibiotic resistance in *Salmonella* isolates from humans with their previous studies in 1968–1969 and with the resistance patterns of *Salmonella* isolates from animals.

Thirty percent of all human isolates were resistant to one or more antibiotic(s). Again, *S. typhimurium* was the most common pathogen and 58 percent were resistant to at least one antibiotic. More than 50 percent of the *S. typhimurium* were resistant to four to five antibacterials. The fraction of all *Salmonella* strains resistant to kanamycin rose from 3 percent to 12.5 percent. When these results were compared with a 1965 national survey conducted by Gill and Hook (Ref. 4), the authors found that the percentage of isolates of all serotypes resistant to ampicillin had increased fourfold by 1973, and the incidence of resistance to tetracycline and streptomycin had approximately doubled. Resistance in *S. typhimurium* had increased from 19 percent to 58 percent of isolates, and resistance to ampicillin has increased from 23 percent to 37 percent. Moreover, the resistance to ampicillin, tetracycline, chloramphenicol, streptomycin, sulfisoxazole, and kanamycin was transferable among the various *Salmonella* strains.

In animals, *S. typhimurium* accounted for 70 percent of the isolates, and 80 percent were resistant to one or more antimicrobial agents. R-plasmids were found in 86 percent of the *S. typhimurium,* and resistance to ampicillin, tetracycline, chloramphenicol, streptomycin, sulfisoxazole, and kanamycin was transferable. Generally, the resistance patterns were similar to those encountered in the *Salmonella* isolated from humans.

The authors conclude that the high incidence of transferable resistance in man and animals suggests that most resistant strains seen today contain complete R-plasmids, and that strains unable to mobilize resistance determinants are less common than was formerly thought. They further conclude that comparison of the resistance of *Salmonella* isolates from humans with that of *Salmonella* from animals shows that tetracycline resistance is greater among the strains from animals, as in the case with sulfonamide and streptomycin resistance. While the resistance to ampicillin is higher in *S. typhimurium* strains isolated from humans than those isolated from animals, the reverse is true for other serotypes. This difference may reflect the greater current use of tetracyclines, sulfonamides, and streptomycin in animals.

Finally, the authors conclude that the survey clearly demonstrates that resistance to antibiotics is increasing in *Salmonellae* isolated from both humans and animals, and since there are great similarities in the resistance patterns of human and animal isolates, it would be useful to know whether the R-plasmids are of a similar nature since this would suggest that animal strains have contributed to the human pool of resistant organisms.

(d) *Smith, H., and J. F. Tucker studies.* Smith and Tucker (Ref. 5) studied the effect of antibiotic therapy on the fecal excretion of *S. typhimurium* by experimentally infecting 3-day-old chicks. There were 3 different treatment regimens studied; 9 different antibiotics were used with experimental groups of 40 during each study. One or two groups in each experiment were fed nonmedicated feed throughout. The following antibacterials were tested: Ampicillin, oxytetracycline, chloramphenicol, furazolidone, neomycin, polymixin, spectinomy-

cin, streptomycin, and a mixture of trimethoprim and sulfadiazine. The regimens were: (1) continuous antibiotic administration in the diet for 61 days at 100 milligrams/kilogram of animal feed (subtherapeutic); (2) continuous antibiotic administration in the diet at 500 milligrams/kilogram of animal feed for 44 days (therapeutic); (3) continuous antibiotic administration in the diet for 9 or 18 days at 500 milligrams/kilogram of animal feed while observing for 65 days.

In each preceding experimental group, except the furazolidone group, when chickens were fed subtherapeutic drugs, the *E. coli* became multiply resistant with R-plasmids having the same pattern of resistance that developed shortly thereafter in the *Salmonella* of the same groups. No antibiotic resistant *Salmonella* were ever isolated from the fecal specimens taken from the chicks fed antibiotic-free diets, although high concentrations of antibiotic-resistant populations always developed in the *S. typhimurium* and *E. coli* from groups fed antibiotics.

Smith and Tucker found that although many of the antibiotics brought about a profound reduction in the concentration of fecal *E. coli*, it was usually short-lived because of the emergence of antibiotic-resistant populations of *E. coli*, even in the group receiving subtherapeutic levels of the antibacterials. Most of the resistance to ampicillin, oxytetracycline, chloramphenicol, streptomycin and spectinomycin are due to R-plasmids found initially in the entire chicken population, with the same patterns of antibiotic resistance (ampicillin, streptomycin, tetracycline, chloramphenicol) which were selected, transferred and subsequently appeared in the *S. typhimurium* populations of each different dietary regimen selected for any one drug.

Although penicillin was not used in the study, the principles that apply to the emergence of transferable drug resistance in this study apply to R-plasmids that emerge from use of penicillin. Further, ampicillin is a penicillin, which in sufficient quantity will produce the effects of penicillin G on drug resistance in Gram-negative bacteria.

Antibiotics have been used to such an extent in certain animal species that organisms that are well adapted to their digestive tract are now drug resistant. The selective pressure of antibiotics is one of the primary factors that results in an increase in the number of organisms carrying transferable drug resistance, and the selective pressure may be from either therapeutic or subtherapeutic antibiotic use. Although the procedures used to gather the information from the AHI chicken study were inadequate according to the current state of the art, nevertheless, the AHI chicken study exemplifies the interaction between the pool of R-plasmid donors and drug-susceptible pathogens in chickens; it also demonstrates the effect of subtherapeutic penicillin pressure on the development of resistance to ampicillin. Other recent literature such as the Smith and Tucker studies and contract studies confirm these findings. The Director concludes that there is no evidence to show that safety hazards do not exist as a consequence of the subtherapeutic use of penicillin in animal feed.

(e) *Kobland, Gustafson study.* Kobland, Gustafson et al. (Ref. 7) of American Cyanamid performed a survey of three major swine producing areas for the Animal Health Institute to determine the extent of the naturally occurring antibiotic-resistant Salmonella reservoir in hogs; subtherapeutic levels of antimicrobials were routinely used in animal feeds in the area. Fecal contents were sampled from market-age hogs obtained from slaughter houses in Pennsylvania, Iowa, and Georgia, and these samples returned to the laboratory for Salmonella isolation procedures. *E. coli* were also isolated to obtain information regarding antibiotic resistance status of indigenous coliforms.

The first survey was made in Lancaster County, Pennsylvania. Out of 151 animals sampled, 54 (35 percent) were positive for Salmonella, and all isolates tested (653) were sensitive to the 10 antimicrobial agents that were tested. Of 31 *E. coli* isolates, 17 were multiply resistant.

In the second study, in Iowa, 26 hogs (10 percent) were positive for Salmonella out of 251 sampled. Examination of 219 isolates yielded 10 (5 percent) resistant isolates, but all from 1 hog. Again, most of the coliforms (*E. coli*) were multiply resistant.

Finally, in the Georgia survey, Salmonella was isolated from 215 (84 percent) out of 256 animals sampled, i.e., 78 hogs (36 percent) carried drug-resistant Salmonella; and of 622 isolates, 145 (23 percent) carried tetracycline resistance singly or with streptomycin.

Four Salmonella serotypes were identified in Pennsylvania, eight in Iowa, and seven in Georgia. The Salmonella strains that were resistant to more than one antimicrobial were able to transfer resistance to an *E. coli* recipient. When the sponsors tested representative drug-sensitive Salmonella isolates for their ability to receive R-plasmids, four *S. worthington* and two *S. newington* isolates acquired resistance after a 24-hour mating. None of 28 other isolates as tested accepted an R-plasmid. Only two samples represented *S. typhimurium*, the most frequently isolated serotype from animal and human sources and a good donor of R-plasimds.

In summary, (i) 40 percent of ceca from animals in Pennsylvania, Iowa, and Georgia contained Salmonella; (ii) None were antibiotic-resistant in Pennsylvania, 4 percent in Iowa, and 23 percent in Georgia; and (iii) none of the Salmonellae from any of the three States were ampicillin-resistant. For *E. coli*, (i) 7 percent of the swine sampled from Pennsylvania were ampicillin-resistant, (ii) 31 percent from Iowa, and (iii) 39 percent from Georgia. Only certain Salmonella serotypes were shown to be good recipients for the *E. coli* R-plasmids in transfer studies done in conjunction with the surveys, and none acquired ampicillin resistance. On this basis, AHI concluded that naturally occurring Salmonella are neither R-plasmid-bearing nor willing R-plasmid recipients.

The survey alone, however, is inadequate to support a conclusion that the background level of drug-resistant Salmonella is not increasing because there is no documentation that the sites selected for sampling provide a random representative sample of the total swine population. The authors explained neither how they determined that the sampled swine had been exposed to antibiotic pressure nor which antibiotics were involved. Of 22 Georgia isolates that were resistant only to tetracycline, not one transferred its resistance, and for this reason, the authors assert that the gene coding for tetracycline resistance was probably located on the bacterial chromosome rather than on a plasmid. This assertion is contrary to current information which indicates that naturally occurring tetracycline resistance is invariably plasmid mediated (Ref. 8). Tetracycline resistance in a bacterial strain can be taken to indicate the presence of an R-plasmid because no evidence has ever shown tetracycline resistance to be chromosomally mediated in naturally occurring strains of enteric bacteria (Ref. 9). The plasmid may, however, be small and not self-transmissible, as was apparently the case in the Gustafson study.

American Cyanamid's in vitro tests for Salmonella R-plasmid recipient activity are also inadequate. Cyanamid tested only "representative" sensitive Salmonella isolates, and four *S. worthington* and two *S. newington* isolates acquired resistance. Although none of the other 28 isolates tested accepted an R-plasmid in these tests, only a single R-plasmid-bearing *E. coli* donor was used, and the compatability properties of the donor R-plasmid were never presented. It is well recognized that certain species of Salmonella are generally neither good donors nor recipients of R-plasmid in the laboratory. The ability of a particular Salmonella to act as a recipient is dependent on the compatability properties of the donor R-plasmid. For example, in recent years most R-plasmids isolated from naturally occurring Salmonella have been of incompatibility groups H and I, and many Salmonella are not good recipients for F II R-plasmids, a common type encountered in *E. coli*. Therefore, without data on incompatability groupings, the Director believes that this aspect of Gustafson's study is of little value.

(f) *Other studies.* Wilcock et al. (Ref. 10), found far greater levels of antibiotic resistance in clinical isolates of *Salmonella typhimurium* (95 percent were tetracycline-resistant) than in isolates of *S. choleraesuis* (18 percent). These strains accounted for 90 percent of the 63 isolates definitely associated with swine salmonellosis. The greater accessibility of *S. typhimurium* to intestinal *E. coli* in contrast to the systemic *S. choleraesuis* infection may explain this difference.

In a survey of 5 Canadian abattoirs by Groves and Barnum et al. (1970, Ref. 11), 20 percent of 462 hogs were Salmonella positive. Tetracycline-resistant Salmonella were found in isolates from 11 of the 94 (11.7 percent) mesenteric lymph node samples of marketed swine, in 2 of 15 (13.3 percent) isolates from the abattoir environment, and in only 1 of 25 (4.5 percent) isolates from a farm supplying the abattoir. Thus, 14 of 134 isolates (10.5 percent) were at least tetracycline resistant. Of the 14 resistant Salmonella, 5 were *S. typhimurium* and 8 were *S. schwarzengrud*. Single or multiple tetracycline resistance was present in all 14 resistant Salmonella. Out of 110 strains studied, 22 were *S. typhimurium*. Other prevalent serotypes included *S. heidelberg, S. muenster* and *S. anatum*. Voogd (1973, Ref. 12) charted various Salmonella serotypes, and a large percentage of resistance was seen in *S. typhimurium* (25 percent in 1971), *S. anatum* (29 percent) and *S. panama* (25 percent), although resistance in other serotypes such as *S. derby, S. infantis, S. dublin,* or *S. chloraesuis* was lower. As mentioned earlier, most surveys have clearly shown an increase in drug-resistant Salmonella in recent years, and the strains surveyed in those studies have obviously encountered R-plasmids which bacteria can accept and stably maintain. This is clearly demonstrated by the results of the AHI studies and the other evidence discussed earlier.

6. *Director's Conclusions.* Questions raised by the CDC reports, and the studies conducted by Ryder, Pocurull et al., Neu et al., and Smith and Tucker (Refs. 1 through 3, and 5) show precisely the same pattern of resistance and in the same sequence that was observed in the *E. coli* and Salmonella isolates from the AHI chicken and swine studies. Resistance occurred in the *E. coli*, and a corresponding pattern of resistance subsequently occurred in the Salmonella after exposure to the R-plasmid-bearing *E. coli*. Despite the absence of antibiotic pressure (in the nonmedicated animals), initially high numbers of resistant *E. coli* in all of the test animals did transfer R-plasmids to the antibiotic-sensitive Salmonella.

Furthermore, because most of the animals in the AHI studies were harboring drug-resistant R-plasmid-bearing *E. coli*. which was contrary to FDA criteria, the studies may be considered invalid for determining the effect of feeding subtherapeutic penicillin on the emergence of drug-resistant Salmonella. Moreover, the procedures used to gather the data on Salmonella prevalence and duration were inadequate. The studies nevertheless demonstrate that the reservoir of R-plasmid-bearing Salmonella increased in direct correlation with the resistance patterns observed in the drug-resistant *E. coli*. These results confirm the results observed in the literature. R-plasmid-bearing bacteria are widespread in the environment, and they can transfer their R-plasmids to pathogens, even in the absence of antibiotic pressure. Under § 558.15, the holders of approved NADA's were required to submit data to prove conclusively that the subtherapeutic use of penicillin in animal feed does not increase the duration and prevalence of Salmonella, and that such use does not contribute to the development of R-plasmid-bearing organisms. Because subtherapeutic use of penicillin contributes both to R-plasmid buildup and transfer, the data lead to the conclusion that the subtherapeutic use of penicillin has not been shown to be safe.

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C. *Compromise of Therapy (Criterion 2 (c))*

1. *Background and criterion.* The 1972 FDA Task Force was concerned that the continuous feeding of antibiotics to animals might compromise the treatment of certain animal diseases. It concluded that additional information was needed, and FDA accordingly determined that epidemiological and controlled challenge studies were to be carried out to determine the relationship of the use of antibiotics in animal feed to the effectiveness of subsequent treatment of animal disease, which is criterion 2(c) of this notice. To answer this criterion with regard to subtherapeutic use of penicillin, the Animal Health Institute submitted two studies. The first, carried out in chickens, involved treatment of a systemic *E. coli* infection by oxytetracycline after subtherapeutic use of penicillin in feed. The second study, in swine, dealt with treatment of a *Salmonella choleraesuis* infection by nitrofurazone, after subtherapeutic use of penicillin in feed.

2. *AHI Compromise of Therapy Study in Chickens.*—(a) *Experimental design.* Day-old-chicks were placed on subtherapeutic levels of penicillin (50 grams/ton) for 21 days. On day 21 the birds were infected by the intramuscular (I.M.) route with *E. coli* at 4.5×10^5 CFU (colony forming units). Subsequent treatment was with oxytetracycline (12.5 milligrams given I.M. for 3 days).

(b) *AHI's summary of the results.* The highest mortality (60 percent) occurred in the group of chickens receiving neither penicillin nor oxytetracycline treatment, as compared with no mortality in the group receiving penicillin in feed and subsequent oxytetracycline treatment. Penicillin-supplmented diets reduced mortality in chickens with systemic *E. coli* infections by 33 percent. The use of oxytetracycline treatment alone was enough to reduce mortality from 60 percent to 13 percent. The penicillin-fed groups showed better weight gain than the control groups.

Based upon the data presented, when mortality, feed consumption, weight gain, and feed efficiency are considered, AHI concluded that the subtherapeutic use of procaine penicillin at 50 grams/ton did not compromise subsequent therapy of artifically induced systemic *E. coli* in chickens, when oxytetracycline 12.5 milligrams I.M. was the therapeutic agent.

(c) *Director's analysis.* The experimental design used was inappropriate to address whether the subtherapeutic use of penicillin in animal feed will compromise therapy in diseased chickens. The establishment of a clinical infection by giving *E. coli* orally in chickens presents some practical problems, whereas challenge via intramuscular injection resulted in a more uniform clinical effect. However, infection by the intramuscular route prevented the interaction, on the intestines, of the infecting organism (*E. coli*) and resident *E. coli*, a combination that is known to be necessary for selection of drug resistance. Therefore, the Director must conclude that this work in chickens presented by AHI fails to address appropriately and to satisfy animal health criterion 2(c). The work provides no evidence that sheds any light on the compromise of therapy issue.

3. *AHI Compromise of Therapy Study in Swine.*—(a) *Experimental design.* Weanling swine were placed on a trial diet (penicillin 30 grams/ton) for 21 days. On day 21 the swine were orally infected with *Salmonella choleraesuis* (2.1×10^9 CFU) via stomach tube, following a 24-hour fast. Treatment was with nitrofurazone (110 parts per million in drinking water) when the first clinical signs of salmonellosis appeared.

(b) *AHI's summary of the results.* The highest mortality (30 percent) occurred in the group of swine receiving no penicillin feed and no subsequent treatment as compared with 10 percent in the group receiving penicillin in feed but no subsequent treatment. No mortality occurred in the groups receiving nitrofurazone treatment, regardless of whether penicillin was absent or present in the diet. The scouring index was higher in the negative control group receiving neither penicillin in the diet nor nitrofurazone treatment, while it was significantly lower in the remaining groups. Weight gain and feed efficiency were higher in the medicated groups than in the control groups.

Although differences in mortality between groups was not significant when other parameters, such as weight gain, feed efficiency, and scour index are observed, AHI concluded that the subtherapeutic feeding of procaine penicillin at 30 grams/ton will not compromise subsequent nitrofurazone therapy of artificially included *Salmonella choleraesuis* in swine.

(c) *Director's analysis.* Any study of compromise of therapy requires a determination of whether the subtherapeutic use of a drug results in an increase in the number of bacteria bearing R-plasmids that are capable of donating these R-plasmids to pathogens. The object of the AHI swine study was ostensibly to determine whether the subtherapeutic use of penicillin would compromise nitrofuran therapy. However, the resistances most commonly found to result from penicillin use in *E. coli* are resistance to ampicillin, tetracycline, sulfonamides, and streptomycin in various combinations. Rarely will the subtherapeutic use of penicillin result in an increased incidence of transferable resistance to nitrofurazone (Ref. 1). For this reason a study that attempts to measure compromise of therapy against nitrofurazone alone will be biased by design against showing a compromise. The nitrofurazone group is useful to show that the disease is treatable by an antibacterial. However, the study requires a group treated with a drug whose resistance is frequently mediated by R-plasmids to measure any compromise of therapy, particularly because penicillin would not be used to treat an *S. choleraesuis* infection. Even though nitrofurazone may be one drug of choice for treatment of *S. chloraesuis* infection in swine, it use alone in the study of compromise of therapy is inappropriate because nitrofurazone resistance is not one that would ordinarily become a problem from penicillin use; moreover, because of questions about carcinogenicity, the Director, in a notice published in the FEDERAL REGISTER of August 17, 1976 (41 FR 34899), proposed to withdraw approval of NADA's for the use of nitrofurazone on the grounds that it has not been shown to be safe.

The study should have been designed with treatment of the disease by a drug to which subtherapeutic use of penicillin may cause increased resistance, e.g., ampicillin or tetracycline, to provide a more accurate reflection of what may occur in the field. This study is of no value in showing that subtherapeutic penicillin feed does not compromise therapy by related drugs such as ampicillin or by drugs to which resistance would commonly occur along with that of resistance on an R-plasmid. For example, ampicillin, tetracycline, sulfonamide, and streptomycin resistance are commonly linked on R-plasmids.

4. *Questions Raised by FDA Funded Research.* Due to the complexity and importance of the compromise of therapy issue, FDA sponsored a study to develop a disease model with antibiotic susceptible organisms in a manner that would provide suspectible pathogenic *E. coli* with the opportunity to interact in the intestinal tract with R-plasmid-bearing organisms and develop drug resistance (Ref. 2). A University of Missouri survey for a tetracycline-susceptible pathogenic *E. coli,* however, failed to locate a susceptible strain in swine, and a compromise of therapy experiment using tetracycline-resistant pathogenic *E. coli* was performed according to the following design.

(a) *Experimental design.* Swine were fed an unmedicated diet and two diets containing subtherapeutic levels of the combination chlortetracycline, sulfamethazine, and penicillin; the investigators then measured the effectiveness of therapeutic levels of chloramphenicol and chlortetracycline.

	Number of animals	Infection with *E. coli*	Oral therapeutic agent (per kilogram of animal)
DIET 1—Unmedicated			
Group:			
1	18	No	None.
2	20	Yes	Do.
3	28	Yes	Chloramphenicol—50 mg.
4	30	Yes	Chlortetracycline—50 mg.
DIET 2—Chlortetracycline (20 g/ton of feed), sulfamethazine (20 g/ton of feed), and penicillin (10 g/ton of feed)			
Group:			
1	17	Yes	None.
2	21	Yes	Chloramphenicol—50 mg.
3	23	Yes	Chlortetracycline—50 mg.
DIET 3—Chlortetracycline (100 g/ton of feed), sulfamethazine (100 g/ton of feed), and penicillin (50 g/ton of feed)			
Group:			
1	14	Yes	None.
2	10	Yes	Chloramphenicol—50 mg.
3	12	Yes	Chlortetracycline—50 mg.

(b) *Director's analysis.* In each diet, chloramphenicol treatment was significantly more effective for the treatment of the disease than was treatment with chlortetracycline. In fact, the results show that chlortetracycline treatment was no more effective than either the untreated control group or the groups fed the combination of subtherapeutic antibiotics in the ration, i.e., the latter were ineffective.

The Missouri study indicates that animal therapy may be compromised where the pathogen is resistant to the antibiotic used for treatment.

5. *Director's Conclusion.* The potential for harm resulting from compromise of therapy is clear, and no evidence has been submitted that adequately addresses the basic issue, the potential for subtherapeutic penicillin use to compromise therapy, since the studies submitted contained design deficiencies. For these reasons, the Director concludes that the sponsors have failed to resolve the issue and thereby show that the subtherapeutic use of penicillin is safe in animal feed.

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6. Optimal Level of Effectiveness (Animal Health Criterion 4). This was originally stated as a separate criterion as follows:

> The optimum usage level for each indication of use of the antibacterial drug at subtherapeutic levels shall not increase significantly with continued use.
>
> Once the optimum level is established, a study shall continue over succeeding generations or populations of animals to determine if this same level continues to yield the same measurable effect.

No data were submitted on this issue for penicillin or penicillin-containing products. The failure to submit these data was in part due to the inability to design such studies that would be meaningful in the 2-year period designated for study. A study begun in 1972 was submitted by AHI which compares the effectiveness of four antibiotics (chlortetracycline, tylosin, bacitracin, and virginiamycin) to a nonmedicated group in swine (Ref. below). The study was conducted at only one location; tests at several locations are necessary to provide any evidence they may have general application to the swine industry. Moreover, the antibiotics were not fed to the swine at graded dosage levels (dosage titration), which is necessary to determine the optimal level of the drug's effectiveness. That is the first step in attempting to address the concerns. Without that evidence, the Director cannot make any determination about the role of R-plasmid-bearing organisms in the continuing effectiveness and safety of subtherapeutic use of any tested antibiotic in animals, including penicillin.

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Langlois, B., G. Cromwell, V. Hays, "Influence of Antibacterial Agents in Feed in the

Incidence and Persistence of Antibiotic-Resistant Members of the Family Enterobacteriaceae *E. coli* Isolated from Swine," final report to Animal Health Institute, April 14, 1976.

D. *Pathogenicity (Criterion 3)*

1. *Background and Criterion.* It is clear that bacterial plasmids contribute significantly to an organism's capacity to produce disease and to survive within the host organism (Ref. 1). The production of enterotoxin, for example, is an essential factor in the pathogenicity of *E. coli* strains of porcine origin, and Smith and Halls (Ref. 2) demonstrated that this property was governed by a plasmid, termed ENT. Similarly, the genetic determinants for enterotoxin production in *E. coli* isolated from calves and lambs have also been shown to be controlled by transmissible plasmid (Ref. 3). Recent studies support the premise that enterotoxin-producing strains of *E. coli* are also responsible for a significant proportion of previously undiagnosed human diarrheal disease (Refs. 4 through 6). Corresponding to these studies in domestic animals, researchers have now shown that the ability of *E. coli* strains of human origin to elaborate enterotoxin is mediated by a transmissible plasmid (Refs. 7 and 8).

In addition to toxins, other plasmid-mediated virulence factors have been described. One of the characteristics of the diarrheal disease caused by enterotoxigenic *E. coli* in man or animals is the ability of large numbers of the bacteria to colonize the small bowel. There is evidence that a surface associated antigen K88, on *E. coli* toxigenic for pigs facilitates colonization since the antigen functions to overcome intestinal motility and other clearing mechanisms (Refs. 9 through 13). Further, Orskov et al. (Ref. 14) showed that K88 production is governed by a transmissible plasmid. A similar antigen, K99, has been described for calves (Refs. 15 through 17). Moreover, these K-antigens play a role in the host specificity of these pathogens. The K88 antigen from porcine isolates is unable to produce adhesion to the calf intestine, and the K99 calf antigen is unable to adhere to the pig intestine (Ref. 15). A similar plasmid-controlled surface antigen has recently been described in a strain of *E. coli*, causing severe human diarrheal disease (Ref. 18).

Another way plasmids can contribute to virulence is exemplified by the colicin V plasmid (Ref. 19). Colicin V is the most common colicin produced by *E. coli*, and pathogenic *E. coli* containing the colicin V plasmid have a greater ability to resist the host species' defense mechanism (Ref. 19). Such *E. coli* also tend to be more refractory to the bactericidal effects of undefined components in serum. In addition, Smith's experiments in chickens and in humans reveal that the colicin V R-plasmid confers on organisms an increased ability to survive in the alimentary tract as well as in the tissue (Ref. 20). On the basis of this evidence, the Director believes that other plasmid-mediated factors that enhance pathogenicity may well be found in the future.

Although pathogenicity is generally determined by more than one factor, the addition of a single specific character to a nonvirulent organism can endow that organism with virulence, and the potential dangers of this character being mediated by a transmissible element are apparent. Because R-plasmids and virulence plasmids can reside in the same bacterial cell, the possibility is increasing that plasmids that contribute to pathogenicity may become more widely disseminated among bacterial species due to the selection of the large reservoir of R-plasmids within enteric organisms.

For these reasons, FDA established Human and Animal Health Safety Criterion 3: "The use of low and/or intermediate levels of an antibacterial drug shall not enhance the pathogenicity of bacteria."

FDA's guidelines required a series of well designed studies to determine if the use of antibacterial drugs in animal feeds enhances pathogenicity of Gram-negative bacilli. First, the sponsors were to determine if plasmids coding for toxin production could become linked to an R-plasmid and be transferred in vitro. If this was demonstrated in germ-free animals, experiments were to be conducted in conventional animals.

Due to the progressional nature of the studies, the Director did not require the sponsors to complete the studies during the time alloted by § 558.15. The sponsors were committed to conduct such studies and to submit reports on the studies at regular intervals. The AHI did submit a study conducted by Dr. John Walton to examine the association of plasmid-mediated toxin production with R-plasmids, and data were also obtained from FDA contracts with Dr. Stanley Falkow and Dr. Carlton Gyles.

2. *Walton Study.* The Walton study (Ref. 21) reported in vitro transfer experiments using a donor organism bearing both the enterotoxin plasmid and R+ factors antibiotic resistance plasmids and a recipient organism that lacks an R-plasmid. Walton concluded that subsequent selection of R+ transconjugants does not select for enterotoxin production.

The Director finds that the study contained major shortcomings in the procedures used, and he rejects Walton's conclusions as inadequately supported. The enterotoxin-producing strains (containing plasmids termed ENT) used in the experiment were inadequately examined for the frequency of transfer of their ENT plasmids and the number of R+ transconjugants tested for ENT transfer (20) was insufficient since only a frequency of 5 percent or greater could be detected. From each mating, 20 transconjugant colonies were pooled and subcultured into 100 milliliters of nutrient broth; then they were grown overnight to obtain cells and supernatant fluid to test for toxin production. However, no positive control was included in the experiment to show that, in screening, 1 known ENT + colony, out of 20 colonies, would produce a positive reaction for toxin production. For these reasons, the Director concludes that the study neither conclusively resolves the issue nor even provides adequate evidence to support the conclusion that selection for R+ transconjugants does not select for enterotoxin production.

3. *Falkow Study*—(a) *In vitro transfer.* On the other hand, Falkow (FDA Contract 73-7210) unequivocally demonstrated that ENT and R-plasmids do cotransfer and that drug selection for the R-plasmid and subsequent clonal screening for ENT was an adequate laboratory tool for detection of cotransfer.

In an in vitro mating, *E. coli* K12 (containing a bovine ENT plasmid, a K-antigen-determining plasmid (K99), and an R-plasmid coding for tetracycline and streptomycin) was crossed to three drug-sensitive *E. coli* K12 recipient strains. The recipient strains were rifampicin resistant, and the donor was rifampicin sensitive. The rifampicin-resistant recipient that received the fetracycline-streptomycin plasmid were recovered on rifampicin-tetracycline drug plates; these recombinant clones were then scored for coinheritance of ENT and K99. Of 225 clones tested (75 from each of the 3 crosses), 2 clones (0.88 percent) received both ENT and K99+. Thus, cotransfer of K99 and ENT plasmid for pathogenicity with the tetracycline-streptomycin drug resistance plasmid was of a low but detectable incidence.

In another in vitro mating study, a bovine enterotoxigenic nonlactose-fermenting *E. coli* isolate (B44) (containing the following plasmids: ENT, K99, and an R-plasmid (R_1) containing genes coding for ampicillin, chloramphenicol, kanamycin, and streptomycin resistance) was crossed with a lactose fermenting strain of *E. coli*, K92 strain 1485. Lactose-fermenting and chloramphenicol-resistant transconjugants were scored for K99 and ENT.

The incidence of K99 plasmid transfer was 3/37 (8 percent) and the incidence of the ENT plasmid transfer was 9/37 (24.3 percent). Furthermore, the incidence of K99, ENT, and R_1 cotransfer was 3/37 (8 percent).

(b) *In vivo transfer.* Falkow fed B44 *E. coli* bearing resistance (R_1), ENT, and K99 plasmids to baby calves, and in vivo transfer of the (R_1) plasmid to indigenous microflora was monitored. In one experiment, ENT plasmid was cotransferred at an incidence of 3/39 (7.7 percent); however, K99 was not transferred. In another in vivo transfer experiment, the ENT was cotransferred at an incidence of 1/88 (1.1 percent) and cotransfer of K99 did not occur. But detection of K99 cotransfer was hampered by the autoagglutination of 50 percent of the transconjugants when slide agglutinations with K99 antisera were performed.

From these experiments, Falkow concluded that possession of an R-plasmid by an enteropathogenic strain does not guarantee cotransfer of ENT or K99; nevertheless, the implications of cotransfer at even a low incidence in the intesti-

nal tract of an animal, should the animal be exposed to the same antibiotics to which the enteropathogen is resistant, has potent public health consequences.

4. *Questions raised by other studies.* (a) Naturally occurring toxigenic strains of *E. coli* are often multiply resistant, and during a recent hospital outbreak of infantile diarrhea in Texas, Wachsmuth et al. (Ref. 23) reported that plasmid-mediated toxin production and multiple antibiotic resistance was demonstrated. Transfer of a 67 × 10^6 and 30 × 10^6 dalton plasmid was associated with the transfer of resistances and enterotoxin production, respectively. Moreover, when antibiotics were used to select *E. coli* K12 recipients from a one-step bacterial cross, all the resistances were concurrently transferred, and 36 percent of these drug-resistant recipient organisms also transferred their ENT plasmids and produced enterotoxin. Clearly, the Director must conclude that R-plasmid transfer can enhance the possibility of ENT transfer and the production of enterotoxin.

(b) Translocation is believed to be the primary mechanism for the dissemination of resistance genes in vivo. Under FDA Contract 223–73–7210, Falkow has been able to show the translocation of antibiotic resistance genes to ENT plasmids in vitro. He also demonstrated that ENT plasmids can acquire resistance genes from R-plasmids if they inhabit the same cell. Ampicillin, sulfonamide, and streptomycin plasmids constructed in vitro by translocation are indistinguishable from such ampicillin plasmids obtained from clinical isolates of *E. coli* and *Salmonella* (Ref. 24).

More recently, Gyles (FDA Contract 223–73–7219) demonstrated the in vivo transfer of ENT plasmids in the intestinal tract of pigs, using the selection of tetracycline-resistant recipient organisms as a basis for screening ENT+ recipient colonies. All of the 35 tetracycline-resistant recipient colonies obtained were shown to bear the ENT plasmid. Gyles also showed that tetracycline resistance and enterotoxin biosynthesis reside on the same plasmid.

5. *Director's Conclusions.* The evidence from both in vitro and in vivo experiments demonstrates that ENT plasmids and R-plasmids can become linked. Only Dr. Walton's study describes data to the contrary; however, his study is inadequate for the reasons discussed. Accordingly, the Director concludes that the existing evidence demonstrates that R-plasmids can increase the pathogenicity of organims, and inadequate evidence has been submitted to prove the contrary.

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E. *Tissue Residues (Criterion 4)*

1. *Background.* FDA has established zero tolerances in tissues of chickens, swine, pheasants, and quail, in milk and eggs for penicillin, its salts and residues. Negligible tolerances of 0.05 part per million exist for the uncooked edible tissues of cattle and turkeys. In all cases the tolerances are a function of the lowest limit that the penicillin assay methods can reliably measure; therefore, the agency in effect permits no residue of penicillin in human food. FDA established these "zero" tolerances because there is no scientific evidence to support a no-effect level for penicillin or its metabolites on the human or animal intestinal flora or on the induction of hypersensitivity. Violative, over tolerance, penicillin residues are regularly reported by the U.S. Department of Agriculture, Animal and Plant Health Inspection Service residue monitoring programs. The FDA followup investigations on the reported violations demonstrate that two routes of administration are primarily responsible for the violations, injection and feed use; and most of the violations are caused by the product misuse, including failure to follow the labeled withdrawal period.

2. *Criterion.* FDA's guidelines requested the following for antibiotics:

Controlled studies * * * to determine whether or not an antibacterial drug used as subtherapeutic levels in the feed of animals results in residues of the parent compound, metabolites, or degradation products in the food ingested by man which are capable of causing (1) an increase in the prevalence of pathogenic bacteria; (2) an increase in the resistance of pathogenic bacteria to antibacterial drugs used in human clinical medicine.

Controlled studies in appropriate test animals shall be conducted to determine whether the consumption of food produced by animals receiving antibacterial drugs will result in:

(a) An increase in the intestinal flora of the prevalence of pathogenic bacteria;

(b) An increase in the degree and spectrum of resistance of the intestinal flora to drugs used in human clinical medicine.

Experimental procedures shall include appropriate consideration of maximum use level, minimum withdrawal time and established tolerances.

In addition, a literature survey shall be conducted to determine the incidence of reports of hypersensitivity resulting from antibacterial drugs in food. The literature survey shall include information regarding hypersensitivity reactions occurring as a result of parenteral or topical exposure to antibacterial drugs as well as those ingested in food. When hypersensitivity has been shown, experiments in appropriate laboratory animals must be conducted to develop estimates of what level of antibacterial drugs in food will cause the production of hypersensitivity.

3. *Data submitted.* Because there is a "zero" tolerance for penicillin and no residues are expected when penicillin-containing products are used in accordance with their labeled withdrawal periods, the sponsors of penicillin were exempted by the Director from submitting the test data. Thus, no data have been provided by the sponsors to show whether the consumption of food produced by animals receiving subtherapeutic levels of penicillin will result in an increase of pathogenic bacteria in the intestinal flora of animals or an increase in the degree and spectrum of resistance of the intestinal flora to drugs used in human clinical medicine.

The firms were required and did, in fact, provide literature data on hypersensitivity reactions to penicillin. These documented the well known alergic and anaphylactic reactions occurring from the penicillins and their degradation products. Human reactions to milk residues after treatment of infections of mammary glands with penicillin was a frequent cause of allergic response; consequently, withdrawal periods from drug usage have been developed before edible products are marketed. One instance (Ref. 1) was cited of a severe hypersensitivity reaction to ingested pork containing penicillin residues.

4. *Director's Analysis and Conclusions.* A study carried out by Katz et al. (Ref. 2) examined the effect of feeding penicillin on the development of residues in edible tissues and the nature of the residues. Although no tissues contained detectable penicillin or its degradation products, penicillin and its degradation products were detected in the crop, proventriculus, gizzard, and duodenum, but not in the small intestine from where it might be absorbed into other body tissues. At the same time chicken feces contained high levels of antibiotic resistant Gram-negative lactose-fermenting organisms (presumably *E. coli*), although no penicillin was present in the feces.

The study, however, raises a question about the safety of penicillin. Although no tissue residues were detected, the feces of broilers fed growth promotant levels of penicillin in their diet exhibited a fairly high percentage of antibiotic-resistant, lactose-fermenting organisms. The resistance was found in spite of the fact that no antibiotic activity could be found in the duodenum of the birds. Accordingly, Katz undertook to investigate the ability of penicilloic acid, one of the major degradation products, to stimulate the development of resistant organisms in the intestinal tract. Groups of birds on three rations were studied, a basal ration, a ration of 50 grams penicillin per ton of feed, and a ration of 50 grams of penicilloic acid of feed. Two resistance markers, tetracycline and streptomycin, were separately incorporated in the agar to act as indicators of resistance.

The percentage of lactose-fermenting organisms in the feces of birds on the basal ration remained relatively low for the period of the experiment, but the birds on the penicillin and penicilloic acid diets showed a markedly higher level of such organisms in their feces. Although the results exhibit some variation due to several experimental factors, the resistance pattern of the lactose-fermenting organisms isolated showed a continuous rise in the percent resistance as reflected in the streptomycin marker. The resistance pattern reflected by the tetracycline marker was more variable, but definitely present. However, the levels of drug resistant lactose-fermenting organisms found in the feces of birds from both the penicillin and penicilloic acid supplemented feeds are at least four times greater than the levels found from birds fed the basal ration. Although not statistically proven, the marked increase in resistance reflected by the marker strongly supports the premise that penicilloic acid can stimulate the development of resistance.

Accordingly, the Director must conclude that feeding subtherapeutic levels of penicillin to chickens may cause an increase in resistant lactose-fermenting organisms. Since the principal lactose-fermenting organisms are *E. coli*, and antibiotic resistant *E. coli* have been demonstrated to transfer R-factors to pathogens, the Director must conclude that the subtherapeutic use of penicillin may contribute to an increase in the prevalence of pathogenic bacteria in the intestinal flora of chickens which is contrary to the criterion established. No data have been submitted to rebut this, and for this reason also the Director must conclude that penicillin has not been shown to be safe.

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V. EFFECTIVENESS

In the FEDERAL REGISTER of July 17 and 21, 1970 (35 FR 11533, 11647, 11650) FDA announced the conclusions of the National Academy of Sciences/National Research Council Drug Efficacy Study Group concerning the penicillin-containing premixes intended for subtherapeutic and therapeutic use in animal feeds. The NAS/NRC evaluated these preparations as probably effective for growth promotion and feed efficiency and concluded that for the remaining claims the products lack substantial evidence of effectiveness that each ingredient designated as active makes a contribution to the total effectiveness claimed for the drug.

The agency concurred with these evaluations, and it provided the manufacturers of these products 6 months to submit adequate documentation of the effectiveness.

Section 512 of the act (21 U.S.C. 360b) requires that a new animal drug have the effect it purports or is represented to have under the conditions of use prescribed, recommended, or suggested in its labeling. For fixed combination drugs, § 514.1(b)(8)(v) (21 CFR 514.1(b)(8)(v)) requires that each ingredient designated as active in any new animal drug combination must make a contribution to the effect in the manner claimed or suggested in the labeling. Furthermore, if in the absence of express labeling claims of advantages for the combination such a product purports to be better than either component alone, the sponsor must establish that the new animal drug has that purported effectiveness. The requirement of effectiveness includes the requirement that the most effective level for each compound be used. In the case of drug combinations for concurrent therapy, the requirement of effectiveness includes the requirement that the dosage of each component is such that the combination is safe and effective for a population of significant size specifically described in the labeling as requiring such concurrent therapy. Therefore, to demonstrate that the penicillin-containing premixes are effective for therapeutic use, the sponsors must submit, in accordance with section 512(d)(3) of the act, substantial evidence consisting of adequate and well controlled investigations, as defined by § 514.111(a)(5) 21 CFR 514.111(a)(5)), including field investigations, satisfying these requirements.

No interested person has ever submitted substantial evidence that the penicillin-containing premixes are effective for the claimed therapeutic uses. For this reason the Director concludes that there is a lack of substantial evidence that the products are effective for therapeutic use in animal feed. Moreover, this action will assure that these levels are not used illegally to replace the subtherapeutic uses that are also being withdrawn.

VI. CONCLUSION

Pursuant to § 558.15, the holders of approved NADA's for penicillin-containing drug products intended for subtherapeutic use in animal feeds have the burden of establishing that this use is safe in accordance with the criteria and guidelines established by that regulation in addition to the basic requirements imposed by the general safety provisions of the Federal Food, Drug, and Cosmetic Act. The Director in this notice has set forth in detail the basis for the criteria and guidelines implementing the regu-

lation and this action. The holders of the approved NADA's have failed to satisfy the legal requirements imposed by the regulations, and they have failed to resolve the basic safety questions that underlie the subtherapeutic use of penicillin in animal feed.

(a) The pool of R-plasmid-bearing organisms is widespread in the environment of man and animals, and antibiotic resistance is increasing in pathogenic and nonpathogenic *E. coli* and Salmonella. The resistance patterns observed in these *E. coli* and Salmonella isolated from animals are similar, and these patterns are similar to the resistance patterns observed in the strains isolated from man. The R-plasmids found in organisms isolated in man and animal are indistinguishable, and common serotypes of these organisms infect both man and animals.

The studies submitted by the holders of approved NADA's through the Animal Health Institute confirm the prevalence of R-plasmid-bearing organisms and the ability of these organisms to transfer R-plasmids to other strains, even in the absence of antibiotic pressure. The AHI studies were also inadequate to measure the duration and prevalance of the Salmonella infections because demonstrably inadequate measuring techniques were used to gather the information.

(b) The potential for harm arising from a compromise of therapy is well documented. None of the studies submitted on compromise of therapy address the fundamental issue—the ability of R-plasmid-bearing organisms to interact and donate these plasmids to other organisms in the intestinal tracts of animals and to acquire resistance to a drug related to the subtherapeutic drug given. Furthermore, no evidence was submitted to show that the effectiveness of subtherapeutic penicillin use over time is not being altered by the development of R-plasmid-bearing organisms.

(c) The evidence demonstrates that R-plasmids controlling pathogenicity, drug resistance, and intestinal motility can and do cotransfer in vitro and in vivo.

(d) Subtherapeutic doses of penicillin and penicillanic acid in chickens causes an increase in drug-resistant lactose-fermenting organisms, e.g., *E. coli*, in their feces. This phenomenon demonstrates a potential for harm, and adequate refuting evidence has not be submitted. In addition, inadequate evidence has been submitted to negate questions on the potential for harm associated with penicillin hypersensitivity and subtherapeutic penicillin use.

(e) Under § 558.15, the holders of approved NADA's were required both to file commitments to conduct studies that would conclusively resolve the safety of the subtherapeutic use of antibiotics in animal feeds and actually to conduct those studies. To ensure compliance with the letter requirement, the regulation required holders of the approved NADA to file periodic progress reports on the studies. The Director is proposing to withdraw approval of all NADA's for which evidence was submitted in accord with § 558.15 purporting to resolve the safety issues, and he is unaware of any sponsor that filed a commitment to conduct the requisite studies but that submitted no evidence. Nevertheless, the Director concludes that the approval of any NADA for which a commitment to conduct appropriate studies was filed but whose holder filed no evidence should be withdrawn on the grounds that the holder of the NADA has failed to establish and maintain records and make reports as required by appropriate regulation.

Additionally, under section 512 of the act, the holders of the approved NADA's have the burden of demonstrating that the products are effective for their indications of use. Based on the evidence now before him, the Director is unaware of any adequate and well controlled investigations demonstrating that the penicillin-containing premixes are effective for the therapeutic uses.

On the basis of the foregoing analysis, the Director is unaware of evidence that satisfies the requirements for the safety of penicillin-containing premixes as required by section 512 of the Federal Food, Drug, and Cosmetic Act and § 558.15 of the agency's regulations. Accordingly, he concludes, on the basis of new information before him with respect to these drug products, evaluated together with the evidence available to him when they were originally approved, that the drug products are not shown to be safe under the conditions of use prescribed, recommended, or suggested in their labeling. The evidence, in fact, indicates that such penicillin use may be unsafe, particularly if the higher or therapeutic levels of penicillin should be used as substitutes for the levels currently used subtherapeutically.

Therefore, the Director announces he is proposing to withdraw all approvals for penicillin-containing premix products intended for use in animal feed whether granted under section 512 of the act or section 108(b) of the Animal Drug Amendments of 1968 (Pub. L. 90-399) on the grounds that they have not been shown to be safe, and lack substantial evidence of effectiveness for therapeutic use. Notice is hereby given to holders of the approvals listed above and to all other interested parties. If a holder of an approval or any other interested person elects to avail himself of an opportunity for hearing pursuant to sections 512(e)(1)(B), 512(e)(1)(C), and 512(e)(2)(A) and § 514.200 (21 CFR 514.200). the party must file with the Hearing Clerk (HFC-20), Food and Drug Administration, Rm. 4-65, 5600 Fishers Lane, Rockville, MD 20857, a written appearance requesting such a hearing by September 29, 1977, giving reasons why approval of the application should not be withdrawn and providing a well-organized and full-factual analysis of the scientific and other investigational data that such person is prepared to prove in support of its opposition to the Director's proposal within 60 days. Such analysis shall include all protocols and underlying raw data and should be submitted in accordance with the requirements of § 314.200 (c)(2) and (d) (21 CFR 314.200 (c)(2) and (d)).

The Director will soon issue a separate notice in the FEDERAL REGISTER proposing to withdraw approval of all tetracycline-containing new animal drug products intended for certain subtherapeutic uses in animal feeds on the grounds that they have not been shown to be safe under section 512(e)(1)(B) of the act and § 558.15. Data addressing the safety and effectiveness issues for the tetracycline component of those products should be submitted at that time.

The failure of a holder of an approval to file timely written appearance and request for hearing as required by § 514.200 constitutes an election not to avail himself of the opportunity for a hearing, and the Director of the Bureau of Veterinary Medicine will summarily enter a final order withdrawing the approvals.

A request for a hearing may not rest upon mere allegations of denials, but it must set forth specific facts showing that there is a genuine and substantial issue of fact that requires a hearing. If it conclusively appears from the face of the data, information, and factual analyses in the request for hearing that there is no genuine and substantial issue of fact that precludes the withdrawal of approval of the application, or when a request for hearing is not made in the required format or with the required analyses, the Commissioner will enter summary judgment against the person who requests a hearing, making findings and conclusions, denying a hearing.

Four copies of all submissions pursuant to this notice must be filed with the Hearing Clerk. Except for data and information prohibited from public disclosure pursuant to 21 U.S.C. 331(j) or 18 U.S.C. 1905, responses to this notice and copies of published literature cited in this notice not appearing in journals designated by 21 CFR 310.9 and 510.95 may be seen in the office of the Hearing Clerk, Food and Drug Administration, between 9 a.m. and 4 p.m., Monday through Friday.

If a hearing is requested and is justified by the applicant's response to this notice of opportunity for hearing, the issues will be defined, an administrative law judge will be assigned, and a written notice of the time and place at which the hearing will commence will be issued as soon as practicable.

The Director has carefully considered the environmental effects of this action, and because it will not significantly affect the quality of the human environment, he has concluded that an environmental impact statement is not required for this notice. A copy of the environmental impact assessment is on file with the Hearing Clerk. Moreover, in a notice published in the FEDERAL REGISTER of May 27, 1977 (42 FR 2739) the Commissioner of Food and Drugs requested data concerning the potential environmental impact of a series of regulatory actions, including this one, designed to restrict

the subtherapeutic use of antibacterials in animal feeds. If the public discussion and information gathered warrant, a comprehensive environmental impact statement will be prepared, evaluating the impact of all the actions as a single program.

NOTE.—The Director has also carefully considered the inflation impact of the notice, and no major inflation impact, as defined in Executive Order 11821, OMB Circular A-107, and Guidelines issued by the Department of Health, Education, and Welfare, has been found. A copy of the FDA inflation impact assessment is on file with the Hearing Clerk, Food and Drug Administration.

(Federal Food, Drug, and Cosmetic Act (sec. 512, 82 Stat. 343-351 (21 U.S.C. 360b)) and under authority delegated to the Commissioner of Food and Drugs (21 CFR 5.1) and redelegated to the Director of the Bureau of Veterinary Medicine (21 CFR 5.84).)

Dated: August 24, 1977.

C. D. VAN HOUWELING,
Director, Bureau of Veterinary Medicine.

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