SCHERING CORPORATION

ANIMAL HEALTH DIVISION

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June 20, 1977

Thomas Raines, D.V.M.
Division-Avian Drugs
Bureau of Veterinary Medicine
Food and Drug Administration
Fishers Lane
Rockville, Maryland 20857

NADA 101-862

Dear Dr. Raines:

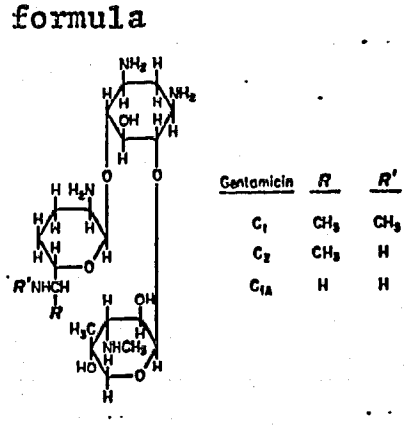
We refer to our new animal drug application for GARASOL^{R} Injection for Chickens and to our telephone discussion on June 13th regarding several points requiring clarification. These are as follows:

Item 1. Make copies of various portions of the NADA concerning EPA.

- These are attached - in triplicate

Item 2. Provide the following:

a. Structural formula



Gentamicin	R	R'
C_1	CH_3	CH_3
C_2	CH_3	H
C_{1A}	H	H

b. Molecular weight
Molecular weight of gentamicin varies because it is a complex of three components, sulfates of gentamicin

C_1, gentamicin C_2 and gentamicin C_{1A} in variable amounts. Generally gentamicin C_1 sulfate is 722, gentamicin C_2 sulfate is 708 and gentamicin C_{1A} sulfate is 694.

c. pH - Gentamicin sulfate in aqueous solution as in GARASOL Injection for chickens has a pH of 3-5.

d. Chemical stability - based on available stability, GARASOL Injection for chickens is stable for 24 months.

e. Define the fungus used in production of gentamicin sulfate.

- o Micromonospora purpurea

Item 3. Support in vitro activity of gentamicin

- The following published paper is attached to support the in vitro activity of gentamicin:

  Hennessey, P.W., et al: In vitro Activity of Gentamicin Against Bacteria Isolated from Domestic Animals. VM/SAC 66, 1118-1122, November 1971.

Item 4. Since the majority of gentamicin is excreted in the first few days of the chicken's life with small amounts then excreted up to five weeks. What are the metabolites.

There is little known about metabolites detected after parenteral administration of gentamicin or other aminoglycosides. It is generally accepted that aminoglycosides are excreted in active form.

In studies done by Waitz and Weinstein, gentamicin serum levels in dogs were assayed by three different methods--microbiological, radioimmunoassay, and C^{14} radioactive assay. Serum levels determined by the three different methods were identical which indicates no metabolism of gentamicin occurred. Levels of gentamicin in urine determined by radioimmunoassay and microbiological assay were similar by both assay methods which further confirms lack of metabolites. Reference: Schering P 4440 - attached.

Item 5. Provide copies of the Freedom of Information summary.

- Copies are attached in triplicate

Item 6. Are any of the data provided in this submission confidential.

- Yes. The material balance involved in the production of gentamicin sulfate veterinary. Submission dated April 5, 1977, page 3, item d.

Item 7. Provide a summary of the drug resistance study as provided with submission dated April 5, 1977.

- Since gentamicin sulfate is being marketed for use in humans, a study was undertaken to determine if a single subcutaneous injection of the recommended dose of gentamicin in chicks has any effect on the sensitivity pattern of Escherichia coli isolates found by cloacal swabs. The Kirby Bauer disc plate procedure was used to determine susceptibility/resistance to gentamicin as well as to neomycin, kanamycin, dihyrostreptomycin, tetracycline and penicillin. A total of 245 E. coli isoaltes from gentamicin treated birds were tested against each antibiotic. The data show that injection of chicks with the recommended dose of gentamicin has no effect on the susceptibility of E. coli to any of the six antibiotics tested. The total susceptibility of isolates to gentamicin was in no way altered.

Item 8. Determine concentration of excreted gentamicin in soil vs. MIC

- Calculated possible gentamicin concentration in the soil is 0.3 mcg./kg. (0.3 ppb). In sensitivity testing by tube dilution methodology, it is impractical to dilute to ppb. Concentrations by tube dilution sensitivity testing may reach 1 mcg./ml. which is in ppm.

Item 9. A small portion of raw materials used in the manufacture of gentamicin sulfate will be discharged to the ecosphere. We state these are carbon dioxide and minute traces of organic solvent. Identify the solvent.

- Chloroform

Item 10. We state that the remaining liquids and solids are discharged to the environment. How and where are they discharged.

- The liquids are barged 40 miles to the ocean under an Interim Barging Permit No. II-PR-104 which covers Spent Broth Wastes from the Environmental Protection Agency.

  The solids are disposed of by dumping in a sanitary land fill in an approved manner.

Item 11. We state that the manufacture of gentamicin requires only 0.6% of fuel used in Puerto Rico and state that this impact on the fuel is insignificant. Explain.

- It is estimated that the production of gentamicin sulfate in Puerto Rico for this product--GARASOL Injection for Chickens--will consume about 0.6% of the fuel used at that manufacture site per year. The small portion of manufactured gentamicin destined to become this marketed product is in this respect considered of insignificant proportion and will not require additional energy beyond that presently allocated.

Item 12. We state that the firm is in compliance with the November 1976 fermentation regulations.

- The manufacturing process for gentamicin sulfate involves only Puerto Rico. We are in compliance with the document published in the Federal Register, Vol. 41, No. 223, November 17, 1976--Part 439 - Pharmaceutical Manufacturing Point Source Category pertaining to Fermentation Products.

Item 13. Provide published data on MICs of gentamicin against a variety of soil bacteria. Support levels of excreted gentamicin vs. soil bacteria. Compare the excreted gentamicin level vs. the 90 dog and cat subchronic oral gentamicin study to show the human safety. The proposed highest level resulting from chicken use is 0.3 mcg./kg. in soil which is 1,000 fold below detectable levels.

- The only published paper (attached) is entitled Sensitivity of Environmental Microorganisms to Antimicrobial agents by P. Van Dijck and H. Van De Voorde published in Appl. Environmental Microbiology, 332-36 March 1976. In this paper sensitivity of different microorganisms considered as typical representatives of microflora of soil and water were tested against gentamicin and 22 other antibacterials. Dilutions were made to 1 mcg./ml. for the sensitivity testing. Practically, dilutions to 1 and 0.1 mcg./ml. are performed but to our knowledge we are not able to locate any dilution factor below. Dr. Van Dijck states that the 1 mcg./ml. is 10-100 times lower than the MICs of strains with ecological importance.

  Our calculations of possibly introducing to the environment via chicken droppings is 0.3 mcg./kg. (ppb). This level is roughly 1,000 fold below any measureable assay sensitivity. It can safely be stated that no organism would be inhibited by the 0.3 ppb, infinitesimal amount of antibacterial.

  Concerning human safety, in 90 day subchronic oral studies with gentamicin sulfate veterinary in dogs and rats, doses as high as 60 mg./kg. were shown to be safe. This calculates to be 200,000 times that level which may be found in soil (0.3 ppb).

Sincerely yours,

ENVIRONMENTAL IMPACT ANALYSIS REPORT

A. Date - June 20, 1977

B.-C. Name of Applicant and Address

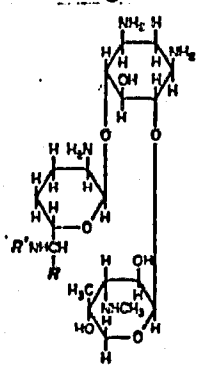
American Scientific Laboratories
Schering Corporation
Madison, Wisconsin 53701

D. Environmental Information

1. Describe the proposed action

a. Purpose of the action -
The applicant proposes to manufacture and distribute GARASOL^{R} Injection (gentamicin sulfate veterinary) recommended for the prevention of early mortality in day-old chickens caused by Escherichia coli, Salmonella typhimurium and Pseudomonas aeruginosa susceptible to gentamicin sulfate.

Gentamicin sulfate veterinary, an aminoglycoside, is a complex antibiotic substance with three components, sulfates of gentamicin C_1, gentamicin C_2, and gentamicin C_{1A}. It is produced from the naturally occurring fungus Micromonospora purpurea, a member of the actinomycetes group. It is a powder, basic in nature and highly soluble in water. Gentamicin solubility in lipids is insignificant compared to its solubility in water. Gentamicin sulfate in aqueous solution as in GARASOL Injection for chickens has a pH of 3-5. Gentamicin is active against gram negative and gram positive bacteria.



Gentamicin	R	R'
C_1	CH_3	CH_3
C_2	CH_3	H
C_{1A}	H	H

b. The environment to be effected if the action is taken -
Manufacture of GARASOL Injection for chickens involves no impact on the environment. Gentamicin sulfate veterinary is produced at the facilities of Schering Corporation in

Puerto Rico. Excipients and packaging supplies are purchased for the final dosage form. No pollutants result from preparation of the final dosage form.

2. Discuss the probable impact of the proposed action on the environment, including primary and secondary consequences.

a. Describe probable adverse and beneficial environmental effects of the use, consumption and disposal of the article that is the subject of the action, including but not limited to the following areas of environmental impact (where applicable):

(1) Pollution (air, water, soil) -
The manufacturing process for gentamicin involves Puerto Rico. Standardized fermentation techniques are used in the manufacture with standard equipment. We are in compliance with appropriate emission certifications for the boilers and fermentors as covered by the Approved Environmental Quality Board (Puerto Rico) Annual Inspection. Manufacture of the final dosage form in New Jersey involves no adverse impact on the environment. We are in compliance with Federal, state and local regulations.

During production of gentamicin sulfate veterinary, air discharge consists mainly of carbon dioxide with minimal traces of chloroform.

The primary impact on the environment may be from excreta from chickens treated with gentamicin. A one day old chick receives a single dose of 0.2 mg. of gentamicin; therefore, the maximum excretion possible is 0.2 mg./bird. The majority of drug is excreted in the droppings the first few days of life though extremely small (immeasurable) amounts may be excreted up to five weeks. The most common poultry housing practice in the U.S. is to place birds in houses at the rate of one bird per 0.75 square foot of floor space.

The most common poultry husbandry practice is to cover the floor with three to four inches of litter (wood shavings). (Wood shavings - 0.75 sq. ft. x 3" weigh 1.3 lbs.)

An eight week old broiler chicken weighing 3.8 lbs. will have, over the eight week period, consumed approximately 7.6 lbs. of feed and 1.9 gallons (16.8/lb.) of water and excreted as urine and feces approximately 16 lbs. or 70% of the total feed and water intake. After evaporation reduces the moisture content by approximately 65%, the droppings would weigh 5.5 lbs. Thus, the weight of litter (1.3 lbs.) and droppings (5.5 lbs.) equal approximately 6.8 lbs. for a broiler over an eight week period. At 6.8 lbs. of manure per bird, it would require 295 birds to excrete one ton of manure. Two hundred ninety five birds would receive approximately 60 mg. of gentamicin which, if totally excreted, would result in a level of 60 mg. gentamicin/ton of manure.

If the manure were spread at the maximum of five tons per acre, there would be 300 mg. of gentamicin into 909,000 kg. of soil. The maximum concentration of gentamicin in soil would be 0.3 mcg./kg.(ppb) which is approximately 1,000 fold below any detectable level.

The preceding calculations assumed the total dose of gentamicin was excreted in droppings and no chemical or biological degradation occurred. Since the calculated maximal concentration of gentamicin in the soil (0.3 mcg./kg.) can not be measured, higher amounts (250, 500 and 1,000 mcg./ml.) of gentamicin were added to dried soil containing sand, silt and clay. Ninety eight percent or more of the initial amount of gentamicin was absorbed to substances in the soil and undetected using the bioassay system. Further tests of the impregnated soil were done to determine any antibacterial activity. Soil with absorbed gentamicin showed no evidence of antibacterial activity. More importantly, a number of studies have shown that gentamicin is readily bound to and thus inactivated by a variety of organic materials including feces and cellulose, as well as diatomaceous earth.

Further, rain fall in soil containing gentamicin chick excreta would dilute the 0.3 mcg./kg. gentamicin to miniscule

quantities. The impact of this use of gentamicin in chicks on soil or water is insignificant.

(2) Solid and liquid wastes (compliance) - ' During production of gentamicin sulfate veterinary in Puerto Rico, the remaining liquids are barged 40 miles to the ocean away from land under an Interim Barging Permit No. II-PR-104 which covers Spent Broth Wastes. The solids are disposed of by dumping in a sanitary land fill in an appropriate manner.

(3) Toxic Substances (heavy metals, pesticides, radiation) - '

Not applicable

(4) Populations (human, animal, plant) - ' No impact on human, animal or plant population is anticipated. Discussed in section D 2 (1). '

(5) Human values, effects - ' Gentamicin sulfate veterinary was fed daily to dogs and rats for 90 days in a subchronic oral study. The no-effect dose of 60 mg./kg. in the dog and rat in relation to the 0.3 mcg./kg. gentamicin which might appear in soil shows at least a 200,000 safety factor to humans. The positive action of this proposal will have no adverse impact on human values, endangered species or places subject to local ordinances.

(6) Food contamination - ' Positive action to this proposal will have no adverse impact on food contamination, considering the miniscule amount of gentamicin which may enter the soil used for food growth.

(7) Natural resources - ' The manufacture of gentamicin sulfate for this action will have an insignificant effect on the natural resources.

(8) Energy - ' Manufacture of gentamicin sulfate for the purpose of this action (GARASOL Injection for chickens) will require about 0.6% of the fuel oil and electrical power used at the manufacturing facility. This will not be in addition to that energy presently allocated.

b. Describe measures taken to avoid or mitigate potential adverse environmental effects.

- Approved ocean disposal wastes; vapor scrubbers for gaseous and/or particulate bearing emissions.

c. Analyze the environmental impact of the manufacturing process of the article that is the subject of the requested action. Include:

(1) An identification of the pollutants expected to be emitted -
During manufacture of gentamicin sulfate veterinary in Puerto Rico a portion of the raw materials used in the manufacture will be discharged into the ecosphere which will have no impact on the environment. We purchase excipients and packaging supplies for preparation of the final dosage form in New Jersey. No pollutants result from preparation of the final dosage form.

(2) A citation of the applicable Federal, state and local emission requirements -
We are in compliance with local, state and Federal requirements. In Puerto Rico we have been issued Interim Barging Permit No. II-PR-104 by the Environmental Protection Agency, which covers the Spent Broth Wastes and we have appropriate emission certifications for the boilers and the fermentors. In New Jersey, the site for preparation of the dosage form, we have been issued Permit No. 0002291.

(3) A certification that such emission complies with said requirements -
We have no annual Environmental Quality Board survey and approval for the current year by such body.

3. Describe the probable adverse environmental effects that can not be avoided.

- Manufacture of gentamicin sulfate is by standard fermentation technique and preparation of the final dosage form is by Good Manufacturing Practice. Waste material disposal and control of all pollutants are in compliance with Federal, state and local regulations.

No adverse environmental effects are expected from indicated use of gentamicin in chickens because of the extremely low gentamicin concentraiton (0.3 ppb) returned to the environment. There is no measurable antibacterial activity observed at this low concentration. This was discussed in section D 2 (1).

4. Evaluate alternatives to the proposed action - There are no alternatives to the proposed action. Raw material alternatives used in manufacture of gentamicin sulfate veterinary would not result in lesser contribution to the environment.

   In ultimate use there is no alternative to the use of gentamicin in chickens. Specificity of gentamicin antibacterial activity in preventing early chicken mortality due to Escherchia coli, Salmonella typhimurium and Pseudomonas aeruginosa reduces economic loss to the chicken producer.

5. Describe the relationship between local short term use of the environment with respect to the proposed action and the maintenance and enhancement of long-term productivity.

   - Short term effects upon the environment are negligible as discussed in section D. Since the short term uses of the environment are not adversely affected by the manufacture and distribution of GARASOL Injection for chickens, the maintenance and enhancement of long-term productivity would also not be affected. The long term benefits consist of improved livability of chickens resulting in a larger meat supply to the consumer.

6. Describe any irreversible and irretrievable commitment of resources that would be involved if the proposed action should be implemented.

   - Based on the manufacture of the product and its use, a portion of the raw materials will be discharged into the ecosphere, mainly carbon dioxide with minimal traces of chloroform. The remainder of the chemical entities are irretrievable while the organic portion of the bio-products are ultimately returned to the natural pool of carbon dioxide and water.

7. Discuss the objections raised by other agencies, organizations, or individuals that are known to the applicant.

   - We know of no one questioning this action.

8. If the proposed action should be taken prior to 90 days from the circulation of a draft environmental impact statement or 30 days from the filing of a final environmental impact statement, Explain why.

- Information presented to our new animal drug application obviates the need of an environmental impact statement. The proposed action makes available to the Poultryman a drug whose safety and efficacy is supported by this new animal drug application.

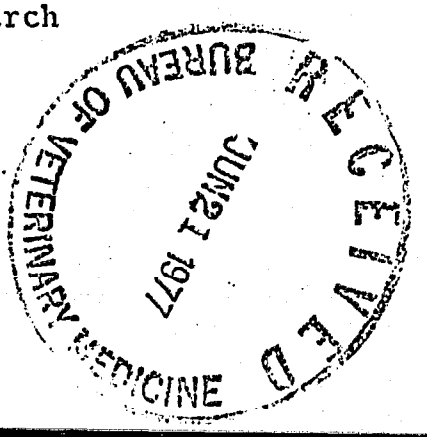
9. Risk-benefit analysis -

- The positive implementation of the proposed action provides to the Poultryman a safe and efficacious product to lower incidence of mortality in treated chickens resulting in a greater amount of poultry available in the meat supply. The benefits of the drug far outweigh the negligible potential risks to the environment presented by GARASOL Injection preparation and use. There is only a minimal potential risk due to the introduction of gentamicin through the poultry droppings or from emission of by-products during manufacture. Calculations of chicken droppings if ultimately used for fertilization of soil would amount to 0.3 ppb. This concentration is much beyond measurable quantities and measurable antibacterial activity. Therefore, it is of minimal consequence. Irretrievable depletion of natural resources due to the manufacture of gentamiicn is so small to be meaningless. Manufacture of gentamicin for this action will in no way demand energy uses beyond that in a normal use.

E. Certification - The undersigned petitioner certifies the information furnished in this Environmental Impact Analysis Report is true, accurate, and complete to the best of his knowledge

6/20/77
Date

Signature
R. A. Berkman D.V.M., Ph.D.
Senior Director, Animal Health Research



SUMMARY

Recent studies (P-4399) in mice demonstrate that aminoglycosides are extensively distributed in tissues and that some of this material is retained in tissues for long times. We have carried out several pharmacokinetic studies which suggest that this is also true in dogs.

The results of two pharmacokinetic investigations in single dogs in which C^{14}-gentamicin was employed may be summarized as follows:

Serum levels of gentamicin are multi-phasic in nature and are consistent with an extensive tissue distribution and some tissue retention.

Serum levels determined by three different assay methods (radioactive, microbiological, and radioimmuno) are identical and suggest that there is either none or very little metabolism of gentamicin in the dog.

Eight days after a single dose of gentamicin, antibiotic was present in significant concentrations in renal tissue and in lower concentrations in twenty different tissue and organ samples.

Excretion of gentamicin in urine was prolonged and multi-phasic in nature.

SCHERING-PLOUGH RESEARCH DIVISION
BLOOMFIELD, NEW JERSEY 07003

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J. Allan Waitz, Ph.D.
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Senior Director
Microbiological Sciences

GHM:JAW:MJW:rm

INTRODUCTION

During the past several years as part of our evaluation of new aminoglycosides and as part of our continuing studies with gentamicin and sisomicin, we have carried out a number of pharmacokinetic studies in several animal species. Results of our studies in mice have recently been reported (P-4399); we report below the results of our studies employing C^{14}-gentamicin in dogs.

MATERIALS AND METHODS

C^{14}-gentamicin was employed as the sulfate adjusted to the base content on the basis of microbiological assay. Dogs were male beagles and weighed approximately 10 kg each. Samples of serum, cage fluid, and urine were obtained by standard methods (P-4310). Samples for microbiological assay were submitted to the Assay Department. Cage fluid samples were assayed against a standard curve prepared in horse serum which had previously been shown to give results similar to standard curves prepared in cage fluid.

Samples for radioassay were prepared and counted in the same way as in previous studies (P-4399). In general, this involved decolorizing with peroxide where appropriate and counting of serum, cage fluid, and urine samples in Scintosol Complete. Tissue samples were digested in Unisol and counted in Unisol Complement.

The gentamicin radioimmuno assay (RIA) employed (New England Nuclear, Biomedical Division) is a competitive assay in which a sample with unknown gentamicin content competes with a known amount

of ^{125}I-gentamicin for a known amount of gentamicin antibody. The antibody is coated onto a plastic tube and after incubation of the sample and iodinated gentamicin in the tube, the tubes are washed to remove all unbound material. The bound ^{125}I-gentamicin is then counted in a gamma counter, and the values compared to a standard curve prepared with samples of known gentamicin content. With proper dilution of the samples, the limit of gentamicin sensitivity of this assay is about 20 ng/ml.

RESULTS

The results of an experiment in which C^{14} labelled gentamicin was given intravenously to a beagle dog are given in Table 1A and Figure 1. The data from the first seven to eight hours are very similar to previously reported data based on microbiological assays for both serum and cage fluid levels (P-4310). The observed peak levels and the initial rapid decline of serum levels, as well as the second slower phase of decline in serum levels are all similar to previous results. The apparent half-life after distributional equilibrium was 55 minutes. The fit of this initial data to a two compartment open model is given in Table 1B. The kinetic parameters are similar to those reported previously (P-4310) or those derived for other data in this report. In addition, similar values have been obtained for kanamycin and amikacin by Cabana and Taggart (Antimicrobial Agents and Chemotherapy, 3:478, 1973).

Because of the sensitivity and precision of the radioassay, we have been able to follow both serum and cage fluid levels for

longer times. One can see (Figure 1) that there is a change in the rate of clearance of gentamicin from serum about eight hours after dosing. During the next 8 or 10 hours, the rate of elimination is considerably slower (half-life of about 154 minutes). A further reduction in serum elimination rate occurs about 15 to 20 hours after dosing. This last phase of elimination has a half-life of about 70 hours, however, the levels are very close to the lower limit of the sensitivity of the assay, and this half-life is very approximate. It is clear, however, that the usual two compartment open model is not adequate to describe these results.

In addition to the serum levels, we have also followed the recoveries of gentamicin from urine. These data are given in Table 1A. The 24 hour recovery of a single dose of gentamicin in urine was similar to previous results with single doses of gentamicin in dogs (66% of the administered dose). A continued excretion of gentamicin was observed during the next 31 days after dosing with an additional 4% of the dose being excreted after the first day. The rate of urinary excretion from the 4th to the 14th day (half-life of three days) is consistent with the observed serum half-life for the fourth phase of serum elimination but after the 15th day, a further reduction in excretion rate occurred (half-life of 10 days) which would suggest the possibility of a fifth serum elimination phase.

While it seems likely that poor urine recovery technique may be responsible for some part of the missing dose, it is questionable

that this could account for the entire 30% of the dose which is missing. We have monitored fecal samples for the first five days after dosing and found only minimal levels of radio-activity which we feel are most probably due to contamination with urine. Additionally, in a separate experiment (unreported data), we have monitored expired CO_2 from mice dosed with C^{14}-gentamicin and have shown that there is no radioactivity in the expired CO_2. Thus, it seems likely that a portion of the administered dose was still present in the dog even after 31 days. Since we have shown in mice (P-4399) and rats (P-4402) that a portion of the dose resides in the tissues of rodents after a single dose for as long as 28 days, it seems most likely that the same is true in the dog.

The data of Wahlig, et al (Int. Clin. Pharmacol., 10:212, 1974) do provide some tissue levels in dogs for as long as 16 days after cessation of a 21 day dosage course, and these data support the idea that significant levels of gentamicin remain in the tissues of dogs, especially the kidneys.

It appears that the most reasonable explanation of our data is that during the initial rapid decline of gentamicin serum levels, gentamicin enters extravascular spaces. Most of this antibiotic returns rapidly to the serum (P-4399) and is excreted, but a small percentage of the antibiotic is retained and is only very slowly released into the serum. Thus, this retained material acts as a reservoir of gentamicin, and its gradual release into serum at varying rates is responsible for the subsequent low serum levels, as

well as the changing rate of decrease in serum levels. This released antibiotic is then excreted in the urine. Wahlig et al (Infection, 3:217, 1975) have recently shown prolonged excretion of gentamicin in man, and it seems likely that a similar phenomenon may be occurring in humans as well. If this theory is correct, then any linear (non-binding) pharmacokinetic model which could be derived to fit the serum data (Table 1B) will be incorrect. The pharmacokinetic parameters obtained from such models will not predict the correct tissue levels. In this report, we have used such models to describe our data, but it should be clear that these models are nothing more than a convenient way of summarizing the data, and no conclusions should be drawn from these models regarding the handling of aminoglycosides. This is, of course, also true of other aminoglycoside pharmacokinetic parameters presented earlier as well.

The cage fluid levels given in Table 1A show that there is a rapid uptake of gentamicin into this fluid with peak levels occurring about 45 to 60 minutes after dosing. The elimination of gentamicin from cage fluid after attainment of peak levels is slower than from serum and appears to be parallel to the third phase of elimination of gentamicin from serum. This kinetic pattern is different from all of the patterns predicted for extravascular compartments by any of the non-binding pharmacokinetic models which we have tried (Table 1B). This difference can be explained by saying that the models are inadequate (as we have

done above) or by assuming that cage fluid is not representative of a normal physiological compartment (see P-report P-4442). Most probably, both of these explanations apply.

We have recently completed an experiment which was designed to verify the tissue-retention hypothesis set forth above. We have not had time to carry out a detailed pharmacokinetic analysis of these data but because of its relevance to this report, the raw data are given. In this experiment, a male beagle dog was dosed intravenously (20 mg/kg) with radioactive gentamicin. Serum levels and urine recovery were followed for eight days at which time the dog was sacrificed, and the residual levels of gentamicin in tissues determined. The serum levels of gentamicin which were determined by radioassay, microbiological assay, and radioimmuno assay are given in Table 2A. In general, the clearance of gentamicin from serum (Figure 2) was similar to the above described experiment. Peak serum levels and initial distributional and post-distributional serum half-lives were consistent with the previous experiment. The third phase of gentamicin clearance occurred at about the same time after dosing and also had a similar half-life. This experiment was carried out with higher specific activity antibiotic (undiluted C^{14}-gentamicin) so that the long term samples would have a greater reliability. In addition, we employed a higher dose and collected more samples; as a result, one can see that the fourth phase of gentamicin clearance starts about 12 hours after dosing and that its half-life is about 50 hours.

Sometime during the second or third day, an additional phase of serum clearance occurs, at which time serum levels remained relatively constant at about 0.015 to 0.020 mcg/ml for the last five to six days of the experiment. Since this level is about the minimum level detectable by either assay, these values are probably not highly accurate. However, since both assays agree fairly closely, it seems likely that there is, indeed, detectable gentamicin in the serum for seven to eight days following this single i.v. dose.

Gentamicin serum levels determined by all three assay methods were generally in close agreement. The small differences which did occur appeared to vary in a random manner and would seem to be the result of experimental error. Because of this agreement, it seems unlikely that any significant amount of metabolism occurs in the dog. A metabolite would have to have the same degree of reactivity as gentamicin in the radioimmuno assay, as well as the same microbiological activity as gentamicin. This seems unlikely in view of the sensitivity of these assays to structural modifications. Levels of gentamicin in urine were determined by radioassay and microbiological assay, Table 2B, and these were also similar by both assay methods.

The percent of the single dose recovered in the urine during the first 24 hours (70%) was very similar to the previous experiment and the excretion of gentamicin throughout the next eight days also occurred at a similar rate (Figure 2). The amount of

gentamicin found in the feces (Table 3B) was very small and could easily result from contamination of the feces with urine. The total percent of the dose eliminated during the eight days by these two routes was about 76% so that 24% or 35 mg must have remained in the dog or have been lost by poor recovery technique.

As can be seen in Table 2C, all of the tested tissues contained some gentamicin eight days after dosing. The levels in the brain, salivary glands, and bone, however, represent the limit of detectability. Renal tissue had the highest levels of antibiotic. Gentamicin levels in the renal cortex were 55 mcg/gm, while the medulla had much lower levels (3 mcg/gm). Thus, about 2 to 3 mg or about 1.5% of the dose was recovered from the kidney. Gentamicin levels in the liver were also relatively high (1 mcg/gm) and account for an additional 0.5 mg of gentamicin. Most of the other organs had lower levels (0.1 to 0.5 mcg/ml) and in total, account for an additional 0.5 mg. If one assumes that the remaining six kg of carcass had an average level of gentamicin similar to that observed in fat, muscle, and bone or about 0.1 to 0.2 mcg/gm, then we can account for an additional 0.6 to 1.2 mg of gentamicin. Thus, we were able to recover about 5 mg of gentamicin in the tissues of this dog.

Since the unexcreted portion of the dose was about 35 mg, we are still missing a significant amount of the drug. Since we have ruled out metabolism, we assume that the missing gentamicin was either excreted in the urine and not recovered or that it was so

tightly bound in the tissues that we failed to detect its presence. This latter explanation is possible since our assay involved a digestion step which may not have recovered all of the antibiotic.

ACKNOWLEDGEMENTS

These studies were carried out by Mr. F. Menzel under the supervision of Mr. E. Moss, Jr. Microbiological assays were performed in the Assay Department under the supervision of Mr. E. Oden. The radioimmuno assays were carried out by Mr. W. Protzman. The surgical implantation of the stainless steel mesh cages was carried out under the supervision of Dr. D. Kentner. The pharmacokinetic analysis of the data was carried out with the assistance of Mr. R. Costello.

The data reported from the above-mentioned studies can be found in the following notebooks:

Table 1 - 7349 x 24

Table 2 - 8571 x 26

TABLE 1A

Gentamicin Serum and Cage Fluid Levels and Urine Recovery following a Single i.v. Dose of 9.4 mg/kg (100 mg) of C^{14}-Gentamicin to a Male Beagle Dog (No. 176)

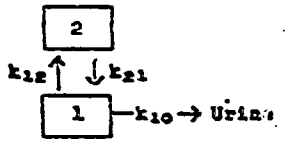
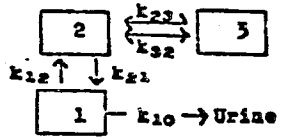
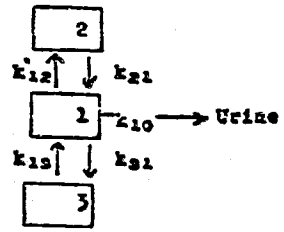
Fluid	Levels (mcg/ml) at Time, Hours after Dosing																					
	1/24	1/12	1/6	1/3	1/2	3/4	1	1 1/2	2	3	4	5	6	7	8	9	10	13	24	30	72	95
Serum	59.4	48.1	39.0	29.5	24.0	20.0	15.5	11.0	7.4	4.2	2.3	1.0	0.61	0.37	0.25	0.18	0.14	0.07	0.03	0.03	~0.01	~0.02
Cage Fluid	--	3.2	5.7	12.1	16.1	18.4	18.8	17.0	14.3	10.6	8.4	5.5	--	4.0	2.5	1.3	1.2	1.2	0.09	0.08	~0.02	~0.01

	Urine Recovery (mg/24 Hours) at Time, Days after Dosing																					
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
Urine	66.6	1.15	0.62	0.32	0.19	0.19	0.11	0.09	0.11	0.06	0.06	0.06	0.05	0.04	0.03	0.02	0.03	0.02	0.03	0.03	0.03	0.02

Urine Recovery (Continued)									Total Recovery/31 Days
23	24	25	26	27	28	29	30	31	
0.02	0.02	0.02	0.02	0.02	0.01	0.02	0.02	0.02	70.0

TABLE IE

Pharmacokinetic Parameters derived from the Serum Levels of a Male Beagle Dog given 9.4 mg/kg of C^{14}-Gentamicin (Table 1A)

Model	Serum Levels[a] Described	Pharmacokinetic Parameters[b] k_{12} min^{-1}	k_{12} min^{-1}	k_{21} min^{-1}	k_{13} min^{-1}	k_{31} min^{-1}	k_{23} min^{-1}	k_{32} min^{-1}	V_1 ml/kg
Two-Compartment Open Model. 	Zero Time to 7 Hours Post Dosing	0.0199	0.0262	0.0406	--	--	--	--	151.2
Three-Compartment Open Model No. 1 	Zero Time to 15 Hours Post Dosing	0.020	0.030	0.046	--	--	0.0019	0.0052	143.1
Three Compartment Open Model No. 2 	Zero Time to 15 Hours Post Dosing	0.020	0.021	0.049	0.008	0.005	--	--	143.1

[a] Serum levels from zero time to 96 hours post-dosing could be described by a four-compartment model, however, since there are six possible variations with no evidence suggesting that any one of these is valid, the parameters have not been calculated.

[b] It was assumed that the i.v. bolus dose was instantaneously distributed in compartment 1. The parameters for the two-compartment model were derived by a computer fit of the data, while the parameters for the three-compartment models were obtained from a graphical fit of the data and manual solutions of the models using the method of Benet, J. Pharm. Sci., 61:536, 1972.

TABLE 2A

Gentamicin Serum Levels following a Single i.v. Dose of 20 mg/kg (146 mg) of C^{14}-Gentamicin to a Male Beagle Dog (No. QCA6)

Assay	Serum Levels (mcg/ml) at Time, Hours after Dosing																		
	1/60	1/24	1/12	1/6	1/3	1/2	3/4	1	1 1/2	2	3	4	5	6	7	8	9	10	11
Radioactivity	110.8	83.6	53.0	50.3	36.9	28.4	20.4	17.5	10.9	7..	3.9	1.8	1.0	0.50	0.32	0.25	0.19	0.091	0.081
Radioimmuno	112	118	70	48	36	26	25	11	6.5	7.1	3.7	1.8	0.97	0.45	0.20	0.23	--	0.12	--
Microbiological	101	71	67	49	39	33	28	15	10	8	4	2	1.0	0.7	0.3	0.2	0.2	0.1	--

	Serum Levels (Continued)															
	12	13	16	19	24	27	30	33	36	48	51	54	57	60	72	78
Radioactivity	0.074	0.072	0.049	0.046	0.028	0.036	0.050	0.035	0.028	0.027	0.029	0.025	0.048	0.044	0.019	0.017
Radioimmuno	0.10	0.13	0.050	0.041	0.032	--	0.028	0.028	0.026	0.017	0.017	0.021	0.017	0.017	0.015	0.018
Microbiological	±	±	±	0	0											

	Serum Levels (Continued)						
	81	84	96	120	144	168	192
Radioactivity	0.016	0.015	0.013	0.016	0.015	0.018	--
Radioimmuno	0.020	0.013	0.016	0.016	0.015	0.012	0.010

TABLE 2B-

Gentamicin Recovery in Urine and Feces following a Single i.v. Dose of 20 mg/kg (146 mg) of C^{14}-Gentamicin to a Male Beagle Dog (No. QCA6)

Assay	Mg Recovered/24 Hours at Time, Days after Dosing							
	1	2	3	4	5	6	7	8
				Urine				
Radioactive	101.8	0.53	0.74	0.34	0.26	0.15	0.13	0.14
Microbiological	104.8	1.2	0.9	0.8	0.5	0.3	0.3	0.1
				Feces				
Radioactive	0.5	0.3	0.2	0.3	0.1	0.2	0.2	--

Assay	Total Recovery, mg
Urine	
Radioactive	104.1
Microbiological	108.9
Feces	
Radioactive	1.8

TABLE 2C

Gentamicin Tissue Levels Eight Days after a Single i.v. Dose of 20 mg/kg (146 mg) of C^{14}-Gentamicin to a Male Beagle Dog (No. QCA6)

Tissue	Gentamicin (mcg/gm)		Whole Tissue Weight, gm		Estimate of Gentamicin recovered, mcg
Tissue	Radioassay	Microbiological Assay			
Kidney			78		
Cortex	54.6	94	"47"[1]		2,566
Medulla	2.7	5	"31"[1]		84
Adrenal	0.41	0	1.7		0.7
Bladder	0.26	0	8.0		2.1
Brain	0.01	0	111.0		1.1
Heart	0.25	0	119.0		30.0
Liver	1.2	0	458.0		550.0
Lung	0.54	0	124.0		67.0
Pancreas	0.18	4	6.2		2.1
Pituitary	0.38	0	0.09		0.03
Prostate	0.11	0	8.3		0.9
Salivary Glands	0.01	0	8.4		0.08
Small Intestine	0.20	0	458.0		91.0
Spleen	0.53	0	262.0		139.0
Stomach	0.17	0	13.5		2.3
Testicles	0.25	3	11.9		3.0
Thymus	0.1	0	9.2		0.9
Thyroid	1.7	0	2.1		3.6
				TOTAL	3,500
Fat (Stomach)	0.24	0			
Fat (Lumbar)	0.39	0			
Skeletal Muscle	0.14	0			
Sternum	0.01	0			
Carcass	--		6000.0		"0.6-1.2"

[1]The kidney was assumed to be 60% cortex and 40% medulla for the purpose of the recovery calculation, on the basis of previous experience.

FIGURE 1. Serum (o) and Wound Fluid (+) Levels of C^{14}-Gentamicin following a Single i.v. Dose of 100 mg (9.4 mg/kg) to a Male Beagle Dog (No. 176)

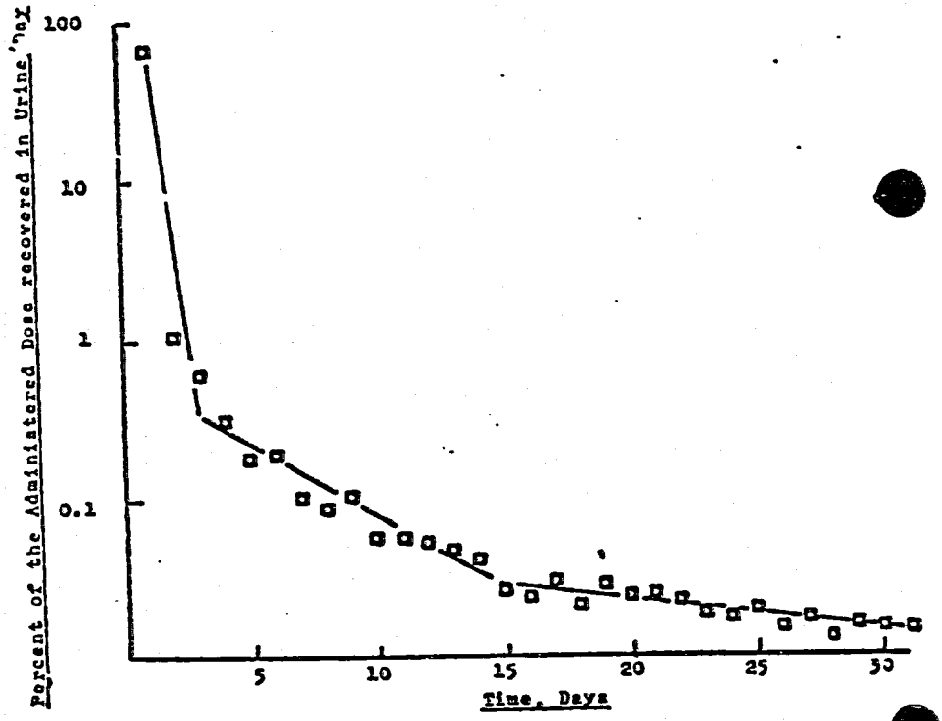
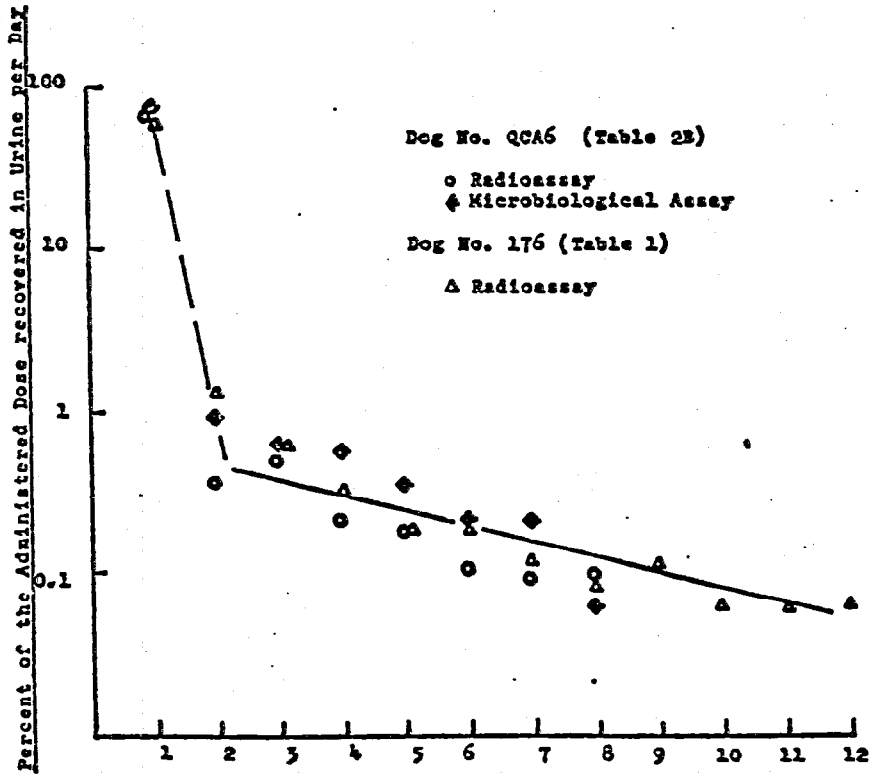


FIGURE 2. Serum Levels of C^{14}-Gentamicin following a Single i.v. Dose of 20 mg/kg (146 mg) to a Male Beagle Dog (No. QCA6)

o Radioassay
+ Radioimmuno Assay
◆ Microbiological Assay



4 8 12 16 20 24 28 32 36 40 44 48 52 56 60
Time, Hours

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Sensitivity of Environmental Microorganisms to Antimicrobial Agents

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The sensitivity of different microorganisms, considered as typical representatives of the microflora of soil and water, was established to evaluate the influence of the nonmedical use of antimicrobial agents on bacterial ecology. Only seven strains, six chemoorganotrophs and one chemolithotroph, could be considered as relatively sensitive to the 21 antimicrobial compounds tested. The other 29 microorganisms may be regarded as resistant to most antimicrobial agents. *Streptomyces* were sensitive to high concentrations of active substances. Broad-spectrum antibiotics showed an effect on environmental bacteria similar to that on human pathogens. Cephalothin stimulated the growth of a *Chlorella* sp. From these experiments, it appears that spilled antimicrobial agents have little chance of causing an alteration in the microbial ecology.

Information about the sensitivity of pathogenic microorganisms to antimicrobial agents, which is nearly complete for type strains, is mainly technical knowledge shared by microbiologists. However, data on minimum inhibitory concentrations on strains of typical environmental origin are rather limited. Some indefinite data are given in *Bergey's Manual* (2) about *Myxococcus* and *Hyphomicrobium*. More precise data are described for bacteria associated with marine algae (1); these studies were performed to obtain pure algae cultures. Many marine strains isolated were sensitive to broad-spectrum antibiotics and to penicillin G. Some antibiotics were also harmful for algae.

Since antimicrobial agents are used extensively outside the medical field, it is extremely important to realize the possible effects of these substances when liberated in the environment. For this reason, some typical representatives were selected from the numerous genera in the bacterial ecosystem to determine their susceptibility to different antimicrobial agents. The work was undertaken mainly to evaluate the possible environmental effect of antimicrobial substances that are used routinely as feed additives in the breeding of farm animals. The conclusions, however, are applicable to the various other fields in which antibiotics are used.

MATERIALS AND METHODS

All antibiotics used were obtained from the pharmaceutical firms that market these products. The purity was guaranteed by these companies, and the desired concentrations were based on these data. The macrolides and peptolides were dissolved at 100 mg/ml in methanol; dimethylformamide was used for solubilization of nystatin; furoxone was used in methanol suspension.

The solvents were tested for possible interference at 0.1% on control plates. Antibiotic concentrations of 100 μg/ml or lower were obtained from an aqueous suspension of the active substance. The concentrated antibiotic solutions were therefore diluted in water. The other antibiotics were dissolved in distilled water and sterilized by filtration through cellulose acetate membranes (0.45 μm).

Rigorous sterility on solid media usually was unnecessary since most media were selective. Thus, disinfection of most antibiotics by the solvents was satisfactory.

The culture methods used were those normally used for the respective organisms. These are specified below. The incubation temperature was 28 to 30 C for all strains.

The common chemoorganotrophic bacteria were cultivated on Trypticase soy agar (Baltimore Biological Laboratories). For all the other strains tested, special compositions were used, which generally are described in classic manuals or papers.

Rhodopseudomonas sp. and *R. sphaeroides* 158 DSM (Deutsche Sammlung von Mikroorganismen) were cultivated on Trypticase soy agar in anaerobic jars (BBL) under artificial light (a 100-W electric bulb, Osram) at 30 C for 4 to 5 days.

Cytophaga johnsonae 425 DSM was cultivated on Trypticase soy agar to which 5% dextrose had been added (sterilized by filtration).

Thiobacillus thiooxidans 504 DSM was developed on the medium as cited in the instruction manual of the culture collection. It contains: NH_4Cl, 0.1 g; KH_2PO_4, 3 g; $MgCl_2 \cdot 6H_2O$, 0.1 g; $CaCl_2$, 0.1 g; sulfur, 10 g; water, 1 liter; pH 4.2 with 1 N HCl. The sulfur was sterilized separately by ultraviolet irradiation.

Hyphomicrobium sp. was grown on the medium studied by Hirsch (7): KH_2PO_4, 1.36 g; Na_2HPO_4, 2.13 g; $(NH_4)_2SO_4$, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $CaCl_2 \cdot 2H_2O$, 9.95 mg; $FeSO_4 \cdot 7H_2O$, 5 mg; $MnSO_4 \cdot 1H_2O$, 2 mg; $Na_2MoO_4 \cdot 2H_2O$, 2.5 mg; agar, 15 g; water, 900 ml; 100 ml containing 2 g of ureum and 1 ml of methanol was added. This solution was sterilized by filtration. The plates were incubated in the dark for 2 days.

Nitrosomonas was cultivated in liquid medium containing: $Na_2HPO_4 \cdot 2H_2O$, 6.45 g; KH_2PO_4, 0.515 g; $NaHCO_3$, 1 g; $(NH_4)_2SO_4$, 2 g; and 1 ml of $FeSO_4 \cdot 7H_2O$, ethylenediaminetetraacetic acid (0.5%), and water, 979 ml. Ten milliliters of $MgSO_4 \cdot 7H_2O$ (0.1%) and $CaCl_2$ (0.03%) was added after sterilization. Five milliliters of the medium was distributed in test tubes (20 by 150 mm) and incubated in a slanted position. Initially the results were estimated after 4 days based on culture turbidity. After 3 weeks, growth was accessed from the formation of NO_2^- using Tromsdorff reagent (Union Chimique Belge).

Growth conditions of *Nitrobacter* were similar to those used for *Nitrosomonas* but using the following medium: $Na_2HPO_4 \cdot 2H_2O$, 3.2 g; KH_2PO_4, 0.272 g; $NaNO_2$, 1.38 g; $MgSO_4 \cdot 7H_2O$, 10 mg; and 20 μg of $ZnSO_4 \cdot 7H_2O$, $CuSO_4 \cdot 7H_2O$, $Na_2MoO_4 \cdot 2H_2O$, and water, 998 ml. After sterilization, 1 ml of $FeSO_4$ (0.5%) and ethylenediaminetetraacetic acid (0.5%) was added. Growth was evaluated after 4 days by turbidity. After 3 weeks an attempt was made to assay NO_3^- with brucine (8), but the results are not reliable since the uninoculated medium also gave a positive result.

Streptomyces were isolated from the soil on the following medium (13): starch, 10 g; $MgSO_4$, 1 g; $CaCO_3$, 3 g; NaCl, 1 g; $(NH_4)_2SO_4$, 2 g; K_2HPO_4, 1 g; agar, 15 g; tap water, 1,000 ml. The sensitivity of the isolated strains was tested on medium containing: peptone, 10 g; meat extract, 5 g; dextrose, 20 g; NaCl, 5 g; water, 1,000 ml; agar, 15 g.

Identification of the bacterial isolates was based on characteristics enumerated in classic manuals (e.g., see references 2 and 12).

The medium for algae was prepared following the description given by Schwoerbel (11); it contained: ethylenediaminetetraacetic acid, 50 mg; $Ca(NO_3)_2$, 20 mg; $CaCl_2$, 18 mg; KCl, 10 mg; $FeCl_3 \cdot 6H_2O$, 3.4 mg; $ZnCl_2$, 20.8 mg; $MnCl_2 \cdot 4H_2O$, 7.2 mg; $(NH_4)_2Mo_7O_{24} \cdot 4H_2O$, 0.13 mg; $CoCl_2$, 0.13 mg; yeast hydrolysate (Nutritional Biochemicals Corp.), 100 mg; sodium acetate, 40 mg; agar, 15 g; in 800 ml. After sterilization 100 ml of distilled water containing 14 mg of KH_2PO_4 and 100 ml of distilled water containing 20 mg of $MgSO_4$ were added. Separate sterilization avoided chemical reaction during heating. The algae were isolated from surface waters. The cultures were cultivated in petri dishes, which were closed with tape to avoid desiccation. The plates were incubated at room temperature (20 to 25 C) in a window facing south but not receiving direct sunlight.

The plates were inoculated from a heavy suspension with a loop. Results were noted after 10 to 14 days, when the control plates showed a good development.

The free-living amoebae were taken from our culture collection (5) and grown on non-nutrient agar. The agar layer contained: NaCl, 0.12 g; $MgSO_4 \cdot 7H_2O$, 0.004 g; $CaCl_2 \cdot 2H_2O$, 0.04 g; Na_2HPO_4, 0.142 g; KH_2PO_4, 0.136 g; water, 1 liter. The surface was seeded with living *Escherichia coli*. The bacterial suspension (10^9 ml) was distributed on the surface of the medium with a folded glass rod.

After a few seconds, the surface was sufficiently dry and a piece of agar, obtained from a culture with heavy growth, was placed on the medium containing the antibiotics. The plates were placed in sealed plastic bags for incubation at 37 C. Inhibition was noted visually by observing the migration of the growth; the cleared zone was easily perceived by observation in indirect light. In doubtful cases, cultures were viewed with an inverted microscope. The growth was followed daily, and the inhibition was established by comparison with the control plate.

RESULTS

The seven *Pseudomonas* strains tested for sensitivity were isolated from mud and may be considered as typical environmental strains. Six of seven *Pseudomonas* reacted like common clinical strains: they were not sensitive (minimum inhibitory concentration) [MIC] > 1,000 μg/ml) to the 21 antimicrobial agents used. Some sensitivity existed to broad-spectrum antibiotics (range, 10 to 100 μg/ml). Flavomycin (10 μg/ml) also inhibited growth of the seven strains. *Pseudomonas* O17 was sensitive to most products tested. This genus appeared to be individually sensitive. Among other criteria mentioned in *Bergey's Manual* (2), the formation of typical pigment on King B medium (Difco) was very striking for all strains (12).

Two strains of *Citrobacter*, one *Klebsiella*, and one *Flavobacterium*, also originating from mud, showed the same high resistance pattern as *Pseudomonas*.

The sensitivity of seven unidentified organotrophic strains from river water (sample taken in the Dijle, Louvain) gave a uniform picture.) Mainly broad-spectrum antibiotics were active (MIC range, between 1 to 50 μg/ml) and the others were inactive.

From additional study of identified and type strains, it appeared that some typical environmental microorganisms were sensitive to antimicrobial agents (Table 1). Only nystatin was inactive (MIC > 1,000 μg/ml) against bacteria. In the genus *Mycoplana*, the two strains *M. bullata* ATCC 4278 and *M. dimorpha* ATCC 4279 were susceptible to most antibiotics used, except the macrolides and penicillins (MIC > 100 μg/ml).

TABLE 1. *Sensitivity of environmental microorganisms to antimicrobial agents*

Antimicrobial agent	MIC (μg/ml) for strain[a]												
	M.b.	M.d.	Hy	Citr. 1	Citr. 2	Flav.	Kl.	Th.	Cy.	Rh.	Hyph.	R.sp.	Nitr.
Broad spectrum													
Tetracycline	1	⊥100	5	100	10	100	100	100	10	⊥100	⊥10	<1	1,000
Polymyxin B								>1,000	100	>1,000	100	10	>1,000
Chloramphenicol	0.5	2	5	5	10	10	100	100	10	1,000	⊥10		>1,000
Aminoglucosides													
Streptomycin	50	100	50	100	1,000	1,000		>1,000	100	100	1,000	<1	⊥1,000
Neomycin	⊥1	⊥1	⊥1	⊥1		<1		1,000	100	100	10	10	>1,000
Gentamicin	<1	<1	<1	<1		<1		1,000	100	100	10	10	⊥1,000
Kanamycin	10	10	10	10		10		1,000	1,000	1,000	1,000	10	⊥1,000
Limited spectrum													
Benzylpenicillin	>1,000	>1,000	>1,000	>1,000	>1,000	1,000	>1,000	1,000	100	1,000	100	100	⊥10
Ampicillin	1,000	1,000	⊥10	>1,000		10		<1	100	>1,000	100	10	100
Cloxacillin	>1,000	>1,000	>1,000	>1,000		>1,000							
Oxacillin								100	1,000	>1,000	10	10	1,000
Cephalothin	1,000	⊥100	⊥100	⊥100		1,000		100	100	>1,000	10	1,000	1,000
Macrolides													
Tylosin	100	100	1,000	>1,000		100		1,000	10	100	100	10	>1,000
Oleandomycin	100	100	100	100	>1,000		>1,000	1,000	10	>1,000	>1,000	100	>1,000
Spiramycin	⊥100	100	⊥100	⊥1,000		100		1,000	⊥10	1,000	100	100	⊥1,000
Virginiamycin	20	100	100	100	>1,000	>1,000	>1,000	10	10	1,000	100	100	1,000
Flavomycin	10	10	1,000	1,000		10		<1	>1,000	<1	>100		1,000
Novobiocin	20	100	100	100	>1,000		1,000	<1	<1	1,000	⊥100	100	>1,000
Bacitracin (U/ml)	100	100	1,000	1,000		10		>1,000	100	1,000	100	10	>1,000
Nystatin	>1,000	>1,000	>1,000	>1,000		>1,000		1,000	⊥1,000	>1,000	>1,000	>1,000	>1,000
Sulfathiazol	>1,000	>1,000	>1,000	>1,000		>1,000		<1	⊥100	⊥100	⊥1,000	100	1,000
Furoxone	>1,000	>1,000	100	100	10	100	10	100	10	1,000	⊥100	100	1,000

[a] Abbreviations: M.b., *Mycoplana bullata* ATCC 4278; M.d., *Mycoplana dimorpha* ATCC 4279; Hy, *Hydrogenomonas* sp.; Citr., *Citrobacter* sp. 1 and 2; Flav., *Flavobacterium* sp.; Kl., *Klebsiella* sp.; Th., *Thiobacillus thiooxydans* 504 DSM; Cy., *Cytophaga johnsonae* 425 DSM; Rh., *Rhodopseudomonas* sp.; Hyph., *Hyphomicrobium* sp.; R.sp., *Rhodospeudomonas sphaeroides* 158 DSM; Nitr., *Nitrobacter* sp. Other microorganisms insensitive to antimicrobial agents used are mentioned in the text. Symbols: ⊥, Partial inhibition; < lower or > higher than given number.

Hydrogenomonas (6) was sensitive to tetracycline and chloramphenicol (MIC = 5 μg/ml). The other antibiotics mentioned in Table 1 inhibited only at 100 μg/ml. If this strain is taken as typical for other species of the genus, a relative sensitivity could be accepted. The strain studied was able to decompose the recalcitrant structure of DDT (6), and this genus may play an important role in the environment.

C. johnsonae 425 DSM was relatively sensitive to many antibiotics (MIC = 10 to 100 μg/ml). The activity of this strain, which decomposes cellulolytic materials, could be altered when large quantities of antimicrobial substances are spilled into the environment.

The photosynthetic strain *Rhodopseudomonas*, very common in mud, was insensitive to the whole series of antimicrobial agents tested and, in this respect, was similar to the *Pseudomonas* genus. In contrast, *R. sphaeroides* 158 DSM, another photosynthetic bacterium from mud, was susceptible to 10 μg of various antimicrobial compounds per ml. Elimination of this microorganism in favor of other bacteria might occur by the presence of active substances in waste material.

Growth of *Hyphomicrobium* sp. was inhibited somewhat by several antibiotics (10 to 100 μg/ml). The multiple mineralization function of this bacterium may be decreased by antimicrobial agents, but only at high levels. The inhibition was mentioned previously in *Bergey's Manual* for *H. vulgare*, but the data were not precise (2).

Nitrobacter was insensitive (MIC > 1,000 μg/ml) to most antimicrobial substances except for penicillin G, which partially inhibited growth at 10 μg/ml. The MICs are based on the biochemical assay of NO_2^- formation. However, after 4 days of incubation, growth appeared to be inhibited from the lack of turbidity in the cultures. Considering this criterion, most agents showed an inhibition. As established, though, the former method is the more reliable test for assaying growth of *Nitrobacter*.

Nitrosomonas appeared to respond similarly to *Nitrobacter*; however, the NO_3^- assay, using brucine as a reagent, may not be trusted completely since control samples of the medium gave also a slight positive reaction. Antimicrobial agents seemed to have little effect on the action of these important microorganisms of the soil.

All eight *Streptomyces* spp., isolated from agricultural land, were sensitive to one or more antimicrobial agents at 1,000 μg/ml (Table 2). Broad-spectrum antibiotics, the macrolides, and others inhibited all strains at high concentrations. None of the strains was inhibited by nystatin, and only one strain was sensitive to flavomycin. The other antimicrobial agents inhibited a varied number of strains. High levels of active substances could change the composition of this major soil microorganism.

TABLE 2. *Sensitivity of eight strains of Streptomyces to 21 antimicrobial agents*

Antimicrobial agent (1,000 μg/ml each)	No. of strains inhibited
Broad-spectrum antibiotics[a]	8
Macrolides[b]	8
Oxacillin	8
Cephalothin	8
Novobiocin	8
Bacitracin (1,000 U/ml)	8
Benzylpenicillin	4
Ampicillin	6
Cloxacillin	4
Virginiamycin	7
Flavomycin	1
Sulfathiazol	4
Furoxone	7
Nystatin	0

[a] Broad-spectrum antibiotics: Tetracycline; the aminoglucosides streptomycin, neomycin, kanamycin, and gentamicin; polymyxin B; chloramphenicol.
[b] Macrolides: Tylosin, oleandomycin, and spiramycin.

The two algae examined may be considered as insensitive to the antimicrobial agents used, but inhibition was still possible at high levels (100 or 1,000 μg/ml). Furoxone inhibited *Chlorella* sp. (4) at 10 μg/ml. Some antibiotic-sensitive algae also were described by Berland (1). An unexpected phenomenon was observed with the cephalothin and ampicillin series. At 1,000 μg/ml a marked growth stimulation was obtained with *Chlorella* sp. in comparison to the control plate. The color was dark green (5 G 5/8) for cephalothin and green (2.5 GY 8/6) (10) for ampicillin, whereas the control appeared as yellowish green (2.5 GY 8/8) (10). Thus, cephalosporin and, to a minor degree, ampicillin interfered with the metabolic pathway of this alga. At 100 μg of cephalothin per ml, the difference in growth between the control and treated cultures was barely perceptible. At 1,000 μg/ml, it was very marked.

The six amoebae strains could be considered as insensitive. Some exceptions were observed, however, three of six strains were sensitive to nystatin (MIC = 10 μg/ml). These results could be expected as the sensitivity of *Naegleria* to amphotericin B (another polyene) is well

known (3). *N. gruberi* 1518 1a and *N. gruberi* LI-S were affected by furoxone and gentamicin, respectively. For amoebae, it may be concluded that they will be scarcely influenced by spilled antimicrobial agents. However, the possibility always exists that they may become affected indirectly by elimination of the feeding substrate.

DISCUSSION

Most chemolithotrophic strains, important for their function in nature, require special growth conditions. Many grow slowly or develop only in symbiosis. Hence a complete survey of the sensitivity of the saprophytic genera would require several years of testing. Nevertheless, the enumerated data could be considered as representative for the bacterial ecosystem, even though the strains studied represent only a small part of the total micropopulation. Additionally, however, most of the strains used in this study were derived from culture collections and have been removed from their natural habitat for varying lengths of time. Thus, sensitivity could be affected by this artificial growth condition. A study in situ would be preferable, but this is very difficult to realize.

The MICs were not accurately established, but obtaining very precise data of inhibition was not the main objective of this work. To evaluate the consequences of environmental contamination by antimicrobial agents, however, rough figures can adequately indicate a possible disturbance in the natural microbial equilibrium.

All bacterial strains examined were gram negative; hence they were inhibited to some extent by broad-spectrum antibiotics. In this sense the Gram property of environmental strains may be extended to their sensitivity in in vitro cultures.

From the data obtained, only seven bacterial strains showed a relative sensitivity to the series of antimicrobial agents used. Compared to the MICs of pathogenic bacteria, this susceptibility is low. Disturbance of microbial ecology and interference in the related role in nature, due to spilled active compounds, would appear to be negligible. Moreover, we have demonstrated by a series of assays, the data of which will be published elsewhere, that the antibiotic activity in surface waters never exceeded 1 μg/ml. This concentration is 10 to 100 times lower than the MICs of strains with ecological importance. The sensitivity of *Streptomyces* spp. must be interpreted in an analogous manner.

Next to possible perturbation of microbial life in nature, contamination by antimicrobial agents also presents the danger of transducible resistance by plasmids. This subject was studied extensively for strains belonging to the normal or pathogenic microflora of man (9). It is accepted that transfer of R factor only occurs within a genus. The genera considered in this study have their natural habitat in the environment; thus plasmid transfer to potential pathogenic strains is highly inconceivable. On the other hand, the transducibility of genetic factors may exist in species that reside in both environments, in man and in nature, e.g., *Pseudomonas*, *Citrobacter*, and *Klebsiella*. Contact of these genera with antibiotics may present an inevitable danger.

ACKNOWLEDGMENTS

This research was made possible by a grant from the Institute for Scientific Research in Industry and Agriculture (I.W.O.N.L.) and with the help of W. Verstraete of the University of Gent, D. Claus of the Deutsche Sammlung von Mikroorganismen (Gottingen), J. B. M. Meiberg of the University of Groningen (The Netherlands), and G. Lambert of the Université Catholique de Louvain, who provided many of the strains mentioned. We thank our colleagues for their cooperation.

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RESEARCH REPORT NO. A-10281
March 30, 1977

N.B. #8425, pp. 44-76

TITLE:
The Effect of Garasol Injection on the Antibiotic Sensitivity Patterns of E. coli Isolates from Chicks

AUTHOR:
Howard J. Bachmann, M.S.

OBJECTIVE:
To determine if a single subcutaneous injection of the recommended dose of Garasol in chicks has any effect on the sensitivity pattern of E. coli isolates found by cloacal swab.

REASON:
This study was designed to answer questions raised by the Bureau of Veterinary Medicine with regard to our petition for the use of Garasol as an injectable in chicks.

RESULTS:
No significant changes in antibiotic sensitivity patterns of E. coli isolates followed injection of chicks with the recommended dose of Garasol. All isolates from treated and untreated chicks were sensitive to gentamicin.

CONCLUSION:
Injection of chicks with a recommended dose of Garasol had no effect on sensitivity patterns of E. coli isolates.

MATERIALS AND METHODS:

A. Animals: One hundred straight-run day-old Hubbard White Mountain Cross chicks were received from Martin's Hatcheries, Lancaster, Pennsylvania. When received, the chicks were randomly assigned to eight groups consisting of 10 birds each and were wing banded for identification as follows:

Group	Numbers
1	401-410
2	411-420
3	421-430
4	431-440
5	441-450
6	451-460
7	461-470
8	471-480

The additional 20 unused chicks were destroyed.

Each group was assigned to a separate section in the same Petersime Brooder with individual heat and light for each group. The birds were maintained in this brooder in an isolation building for two weeks then transferred intact to Model 4F Petersime growing cages to allow them sufficient room during the remainder of the trial period.

All birds received well water and unmedicated starter mash ad libitum during the trial.

B. Treatment and Dosage:

Drug: Garasol Solution - Brand of gentamicin sulfate veterinary - 50 mg/ml. 6PTX3P54574 Expiration May 1979.

This drug was diluted 1:50 by adding 1.0 ml to 49.0 ml of sterile saline.

Each chick was injected with 0.2 ml (0.2 mg gentamicin) of the diluted drug given subcutaneously in the neck region just behind the skull.

C. Test Design and Procedure: After the random distribution the groups were divided into four treated and four control groups of ten birds each. Groups 1 thru 4 were untreated controls and groups 5 thru 8 were injected with the recommended dose of Garasol.

Cloacal samples were obtained from ten birds from control groups and ten from treated groups at 0, 7, 14, 21 and 28 days post treatment. Birds were selected for sampling at each sampling time by use of tables of random numbers.

D. Laboratory Procedures: Cloacal samples were streaked directly on desoxycholate agar (Baltimore Biological Laboratories) in 100 mm x 15 mm plastic petri dishes

(Falcon Plastics) so that individual colonies would be obtained. All plates were incubated for 18-24 hours at 37°C.

After incubation five colonies typical of E. coli (bright red indicating lactose fermentation) were selected from each plate (representing each bird). Each colony selected was suspended in 2.5 ml of Mueller-Hinton Broth (BBL). From this broth a triple sugar iron (TSI) agar slant was inoculated and incubated with the broth at 37°C for 18 hours. After incubation all broth suspensions confirmed to be E. coli by a typical reaction of the accompanying TSI were used to determine sensitivity patterns to gentamicin (Gm), neomycin (N), kanamycin (K), dihydrostreptomycin (DS), tetracycline (Te) and penicillin (P) by standard disc susceptibility tests as described by Bauer, et. al. (1). Mueller-Hinton agar plates used for the Kirby-Bauer procedures were prepared on Monday and inoculated on Thursday. These were 100 mm x 15 mm plastic petri dishes to which 25 ml of agar was added to give a depth of 4 mm.

Each broth culture was streaked on an agar plate using a sterile cotton tipped swab. One disc containing each of the antibiotics to be tested was placed on the plate. All plates were incubated overnight at

37°C. Zone sizes in millimeters were recorded for each antibiotic disc on the following day.

Isolates were considered sensitive if the zone around antibiotic discs were: Gentamicin (12 mm or more), Neomycin (17 mm or more), Kanamycin (17 mm or more), dihydrostreptomycin (18 mm or more), tetracycline (19 mm or more) and Penicillin (22 mm or more). All other isolates were considered resistant to the antibiotic in question.

RESULTS:

Table I summarizes the results of antibiotic sensitivity tests on E. coli isolates obtained. A total of 245 E. coli isolates from untreated control birds and 245 from treated birds were tested against each antibiotic.

All of the isolates obtained from either untreated control or treated birds were susceptible to gentamicin and kanamycin. Only one isolate from untreated control birds and one from treated birds proved to be resistant to neomycin. None of the isolates were susceptible to penicillin. Most strains of E. coli isolated from either untreated control or treated groups were susceptible to dihydrostreptomycin (DS) and tetracycline (Tc). However, at most sampling periods isolates which were resistant to dihydrostreptomycin and/or tetracycline were recovered from both the treated and untreated groups.

DISCUSSION:

All strains of E. coli isolated from both untreated control and treated groups at every sampling interval were susceptible to gentamicin and kanamycin. With the exception of one strain from the untreated control and one from the treated birds, the same was true of neomycin. This indicates that within the conditions of this experiment the susceptibility of isolates to these three antibiotics was not altered.

The total resistance of E. coli isolates to penicillin was not altered during this trial.

There were strains isolated from both untreated control and treated birds which were resistant to dihydrostreptomycin and/or tetracycline. These strains were isolated at most sampling periods and more were obtained from the untreated control birds than from the treated birds.

CONCLUSION:

Injection of chicks with Garasol at the recommended dose had no measurable effect on the susceptibility patterns of E. coli to any of the drugs tested. In particular, the total susceptibility of isolates to gentamicin was in no way altered by treatment at the recommended dose.

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GARASOL/Chick EIAR Summary

I. Describe Proposed Action:

A. Concise Description:

Schering Corporation, Animal Health Products, proposes to manufacture and market GARASOL Injection, brand of gentamicin sulfate veterinary, as an aid in the prevention of early mortality in day old chickens caused by bacteria susceptible to gentamicin including E. coli, Enterobacter aerogenes, Proteus mirabilis, Salmonella typhimurium and Pseudomonas aeruginosa.

B. Chemical/Physical Properties of New Drug

Gentamicin sulfate veterinary is an aminoglycoside antibiotic, derived from Micromonospora purpurea, a member of the Actinomycetes group and originally isolated from a domestic soil sample. It is a powder, basic in nature and highly soluble in water. Gentamicin solubility in lipids is insignificant compared to its solubility in water. Aqueous solutions are stable over a wide range of temperatures and pH.

C. Uses and Benefits of Proposed Action

Early mortality in chicks creates a major economic loss to the poultry industry and if uncontrolled may lead to consumer price increases. A single subcutaneous injection of 0.2 mg. gentamicin in day-old chicks provides the necessary activity to eliminate many debilitating infections, which if untreated could result in death.

D. Potential Market

Based on current estimates there are approximately 300 million pullets and 3 billion broilers raised annually in the United States. Optimistically we estimate approximately 700 million chicks or slightly more than 21% of the population may be treated with the recommended dose of GARASOL Injection.

II. Manufacturing Impacts

A. Identify Pollutants Expected to be Emitted

The manufacture of GARASOL Injection for chicks involves no impact on the environment. Gentamicin sulfate veterinary is produced at the facilities of Schering Corporation in Puerto Rico. Excipients and packaging supplies are purchased. No pollutants result from preparation of the dosage form.

B. Citation of Applicable Federal, State and Local Emission Requirements and

C. Certification that Schering Complies with These Requirements

Schering complies with all local, state and federal environmental requirements. Prior to installation of new plant equipment in production, local and state agencies are informed and after inspection, permits are obtained.

D. Identify any Non-renewable or Scarce Resources Used by Manufacturing Process

There are no scarce or non-renewable resources utilized in the preparation of gentamicin sulfate veterinary or the subsequent manufacture of GARASOL Injection.

III. Introduction into the Environment

A. Drug excretion and concentration.

A one day old chick receives a single dose of 0.2 mg. of gentamicin; therefore, the maximum excretion possible is 0.2 mg./bird. The majority of drug is excreted in the droppings the first few days of life though extremely small (immeasureable) amounts may be excreted up to 5 weeks. The most common poultry housing practice in the U. S. is to place birds in houses at the rate of one bird per 0.75 square foot of floor space.

The most common poultry husbandry practice is to cover the floor with three to four inches of litter (wood shavings). (Wood shavings - 0.75 sq. ft. x 3" weigh 1.3 lbs.) An eight week old broiler chicken weighing 3.8 lbs. will have, over the eight week period, consumed approximately 7.6 lbs. of feed and 1.9 gallons (16.8/lb.) of water and excreted as urine and feces approximately 16 lbs. or 70% of the total feed and water intake. After evaporation reduces the moisture content by approximately 65%, the droppings would weigh 5.5 lbs. Thus, the weight of litter (1.3 lbs.) and droppings (5.5 lbs.) equal approximately 6.8 lbs. for a broiler over an eight week period. At 6.8 lbs. of manure per bird, it would require 295 birds to excrete one ton of manure. Two hundred ninety five birds would receive approximately 60 mg. of gentamicin which, if totally excreted, would result in a level of 60 mg. gentamicin/ton of manure.

If the manure were spread at the maximum of 5 tons per acre there would be 300 mg. of gentamicin per acre. Common practice of plowing or discing the manure into the top six inches of soil would distribute the 300 mg. of gentamicin into 909,000 kg. of soil. The maximum concentration of gentamicin in soil would be 0.3 mcg./kg. (ppb) which is approximately 1000 fold below any detectable level.

B. Geographical Use Pattern

Usage will be mostly in areas of intensified poultry production which include Delmarva, Pennsylvania, Maine, Arkansas, Texas, Georgia, Alabama, North Carolina, South Carolina, Mississippi, Florida, Illinois, Indiana, Ohio, Iowa, Wisconsin, Minnesota, California, Oregon and Washington. It is anticipated the southeastern states (Georgia, Alabama, North and South Carolina, Mississippi and Florida) would have the heaviest use because of the number of broiler chickens raised. Total material to be used for this purpose is approximately 140 kg./year which will treat 700 million birds. This 140 kg. would account for approximately one half of all gentamicin used in animals (total gentamicin used in 1975 was 159 kg.)

IV. Environmental Fate

The preceding calculations assumed the total dose of gentamicin was excreted in droppings and no chemical or biological degradation occurred. Even under those assumptions, the amount of gentamicin in soil would be undetectable. If the same amount of gentamicin was applied to the soil in manure twice a year for 10, 50, or 100 years the level would still remain far below any detectable level.

Several studies were designed to examine the availability, activity, persistence, and movement of gentamicin in soil. Since it is impossible to work with the low levels calculated to be in soil, levels greater than 10,000 fold the calculated level were used in these studies.

A. Availability

Gentamicin was added directly to five gram soil* samples to yield concentrations of 1000, 500 and 250 mcg./gram. Routine extraction techniques were performed to recover the drug from the samples. A microbiologic assay procedure was then utilized to determine the percentage of "active" gentamicin extracted.

Between one and two percent of the gentamicin was extractable and microbiologically active using routine assay procedures. Ninety eight percent or more of the initial amount of gentamicin added was absorbed to substances in the soil and undetectable using the bioassay system.

The results indicate gentamicin is one of a group of basic antibiotics that is readily absorbed by negatively charged or colloidal materials in the soil.[1,2]

Gentamicin (mcg./gram) in Soil

	1000	500	250
Mean % Recovered	1.20[a]	1.14	1.01

[a]Assay sensitivity:0.04 mcg./ml. buffer

* All studies were conducted with 24 hour dried soil containing 64% sand, 16% silt and 20% clay.

B. Antibacterial Activity

A modified plate count procedure was designed to detect antibacterial activity of absorbed gentamicin. Using routine procedures, gentamicin was extracted from soil samples containing 1000, 500, 250, 100, 50, and 0 mcg./gram of gentamicin. The extracts were assayed by the standard microbiologic assay procedure to determine the percent recovery of free or extractable gentamicin. With this procedure, an estimate of the percent of adsorbed gentamicin remaining in each soil sample was obtained. The extracted soil samples were autoclaved and known numbers of S. epidermidis ATCC 12228 (10^{11}) were added to each sample. This organism is a primary assay organism and is sensitive to 0.04 mcg./ml. of gentamicin in buffer. Following a suitable period of exposure (60 minutes) at room temperature, saline was used to extract and recover the bacteria from each sample. The number of bacteria recovered was determined by standard plate count procedures.

Antibacterial activity of adsorbed gentamicin equivalent to 0.04 mcg./ml. or more of free gentamicin should have resulted in a reduction in the numbers of S. epidermidis 12228 recovered per sample.

The results were as follows:

Initial gentamicin concentration (mcg./gr.)	Gentamicin % adsorbed[a]	S. epidermidis 12228 colonies recovered/5 gr.soi
1000	99.07	2.8×10^{10}
500	98.92	3.9×10^{10}
250	99.00	6.6×10^{10}
100	>99.60	3.9×10^{10}
50	>99.60	4.0×10^{10}
0	Neg.[b]	6.5×10^{10}
0	Neg.	8.6×10^{10}
0	Neg.	2.0×10^{10}
0	Neg.	5.5×10^{10}
0	Neg.	1.0×10^{10}
0	Neg.	3.6×10^{10}

[a] Assay sensitivity: 0.04 mcg./ml. buffer

[b] No evidence of antibiotic activity.

The number of bacteria recovered from soil samples containing adsorbed gentamicin ranged from 2.8×10^{10} to 6.6×10^{10}. The number of bacteria recovered from control samples ranged from 1.0×10^{10} to 8.6×10^{10}. The number of bacteria recovered from samples containing adsorbed gentamicin clearly fall within the range of bacteria recovered from control samples. No decrease in the number of bacteria in treated samples as compared to control samples occurred. Similar results have been reported for other antibiotics.[3]

The results show gentamicin does not retain its antibacterial activity when adsorbed to soil colloids.

C. Movement in the Soil

One and a half micrograms of tritiated gentamicin (specific activity - 0.911 ci/mole) was slurried onto the top inch of a twelve inch soil column (diameter - 0.84 inches). Three hundred and seventy six milliliters (6.6 acre inches) of distilled water was dripped continuously on the top of the soil column. Eluants taken hourly from the bottom of the column were counted directly in a liquid scintillation counter. None of the eluates contained counts substantially different from background levels.

The column was then divided into one inch segments which were lyophilized. From each segment five samples weighing 0.5 grams each were oxidized to CO_2 and H_2O and assayed for tritium. The assay results showed 90% of the tritium was retained in the top one inch of soil. The remaining 10% of tritium was retained in the second inch of soil. None of the soil samples lower than two inches in the column contained counts substantially higher than background.

The results of this test indicate that gentamicin is not highly motile in soil. Therefore, the introduction of gentamicin to grondwater via leaching is a remote possibility.

D. Stability

A study designed to determine the stability of free gentamicin was based on percent recovery of drug from soil over a period of time.

Gentamicin was added to fifty gram pre-sterilized and non-sterilized soil samples to yield concentrations of 500 mcg./gram. At two week intervals the moisture content of the samples was adjusted to 50% of the water saturation capacity of the soil. All samples were maintained at room temperature. Samples were assayed for residual gentamicin at 0, 4, 10, 20, and 31 days using a routine microbiological assay. The results are as follows:

Gentamicin Stability in Soil

	Non-Sterilized Soil Zones of Inhibition		Pre-Sterilized Soil Zones of Inhibition	
	(mm)	% Recovered	(mm)	% Recovered
0 day	26.5	1.83	27.7	2.43
4 days	26.7	1.71	26.7	1.67
10 days	27.2	1.61	28.0	1.92
20 days	25.8	0.88	26.3	0.99
31 days	24.5	0.80	25.1	0.91

These data show that gentamicin activity decreased with time in both pre-sterilized and non-sterilized soil. The loss of activity may be due to biological deactivation (enzymatic phosphorylation, adenylation, etc.) or chemical inactivation or binding. At day 31, the gentamicin activity is 43% and 37% of day 0 for the non-sterile and pre-sterile soil samples respectively

Summary: Introduction into the Environment

Calculations on drug excretion and introduction into the environment based on normal poultry husbandry practices indicate the level of gentamicin that could be found in soil would be less than 0.3 ppb. Studies showed 98% of gentamicin added to soil was adsorbed and less than 2% could be extracted. Evidence of similar adsorption of gentamicin to a variety of substances has been documented by other investigators.[4,5,6]

V. Effects

A. Acute and Chronic Toxicity Data in NADA

1. Day-old chicks were randomly divided into 4 groups. Each group received a single 0.2 ml. subcutaneous injection containing either 0, 0.2 (recommended dose), 1:0 or 10.0 mg./chick. Mortality, weight gain, feed conversion, serum chemistries, hematology, and gross and histopathology data were recorded at various intervals during the study.

   Data showed the LD-50 was approximately 425 mg./kg. Mortality in the 0.2 and 1.0 mg. groups was less than in controls. Mortality increased and daily weight gain decreased in the 10 mg. group. No adverse effects were noted in the 0.2 and 1.0 mg. groups. No differences were observed in the serum chemistries, hematology, gross or histopathology.

2. Day-old broiler chicks were randomly divided into 4 groups. Each group received a single 0.2 ml. subcutaneous injection containing either 0, 0.2, 0.4, or 0.6 mg. gentamicin. Individual bird weights were taken at one day of age and weekly for 8 weeks. Body weights, feed consumption and feed conversion were determined for each group. Data showed gentamicin does not adversely affect weight gain, feed consumption or feed conversion through 4, 5, and 8 weeks post injection. The study was terminated at 8 weeks.

B. Ninety-six (96) Hour TC-50 for Bacteria Algae, and Fish Species or Soil Organism Such as Earthworms

GARASOL Injection is recommended for one time use in day-old chicks. It is not intended for continuous administration. (In addition, it has been shown that although gentamicin is partially excreted in an active form, it is degraded or inactivated shortly after entering the soil). For these reasons the above study is deemed unnecessary since gentamicin will not come in contact with the cited organisms in an active form.

C. Phytotoxicity to Plants

See B above.

R. N. Berkman

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10/8/86
Date

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SCHERING CORPORATION MEMO

TO: Files

FROM: Susan R. Spreat

SUBJECT: Environmental Impact of Gentamicin

REFERENCE: Notebook 8831, pages 23-75

DATE: October 8, 1976

COPIES:

In response to an inquiry by the Environmental Protection Agency concerning the environmental impact of gentamicin following excretion from treated animals, a series of studies was completed investigating the behavior of gentamicin in soil. The studies covered the following areas: availability, antibacterial activity, and stability. Soil containing 64% sand, 16% silt, and 20% clay was used in all studies. Detailed methodology may be obtained from Notebook #8831, pp. 23-75.

I. Availability

The purpose of this study was to determine the extent of adsorption of gentamicin to soil.

Design

Gentamicin sulfate (10,000 µg/ml working standard) was added to 5 gram soil samples to yield the following concentrations: 1000, 500, and 250 µg/ml. Five milliliters of 0.1 M PO_4 buffer pH 8.0 was added to each sample. The samples were mixed on vortex mixer - maximum speed 0 then centrifuged for 15 minutes at 20,000 rpm. The final supernatant was assayed against a standard gentamicin curve prepared in 0.1 M PO_4 buffer. The concentrations of the buffer curve were: 0.64, 0.32, 0.16, 0.08, 0.04, 0.02, 0.01, 0 µg/ml. A reference solution containing 0.05 µg/ml gentamicin was prepared in 0.1 M PO_4 buffer pH 8.0 for use in the assay.

Assay Method

The standard and unknown samples were assayed using a microbiologic cylinder plate procedure. The assay organism was S. epidermidis ATCC 12228. Media 11 (agar-special agar noble) was used for the 6 ml base layer and the 3 ml seed layer. The media was adjusted to pH 8.0 and inoculated with a bulk suspension of S. epidermidis ATCC 12228 (O.D. 0.6). Six stainless steel cylinders were placed on the agar at 60° intervals. Three alternate cups were filled with standard or unknown samples. The remaining cups were filled with reference solution. Three 100 x 20 mm petri dishes were plated per standard or unknown sample.

The plates were incubated at 36°C overnight. The resulting zones of inhibition were measured using a Fisher-Lilly Zone Reader. Means were computed for nine readings per sample. The mean of the reference standard was

used to correct the sample means for plate variation. The zone diameters (mm) of the standard curve were plotted versus gentamicin concentration (µg/ml) on 3 cycle semilog paper. The concentration in µg/ml buffer of the unknown samples was read from the resulting curve. Percent recovery was calculated for all unknown samples (µg/ml recovered from homogenate/theoretical µg/ml of homogenate).

Results

The results for three separate trials were as follow:

	Gentamicin (µg/gr)		
	1000	500	250
% recovered	1.08	1.40	1.28
	1.58	0.94	1.00
	0.93	1.08	0.75
$\bar{X}$	1.20	1.14	1.01

The mean percent recovered for the three concentrations (1000, 500 and 250 µg/gr) of gentamicin tested were 1.20, 1.14 and 1.01 respectively.

Between 1 and 2 percent of the initial amount of gentamicin was extractable by routine procedures and biologically active using a microbiological bioassay technique. The results indicated that gentamicin was highly adsorbed (>98%) to elements in the soil.

II. Antibacterial Activity

The purpose of this study was to test "adsorbed" gentamicin for antibacterial activity.

Design

Soil samples containing 1000, 500, 250, 100, 50 and 0 µg/gr gentamicin were extracted and assayed using the procedures previously described. Percent gentamicin recovered and percent adsorbed were calculated using the assay results.

The remaining soil pellets were autoclaved for 3 minutes (media cycle). One milliliter of a 24 hr. bulk suspension of S. epidermidis ATCC 12228 (approximately 10^{9} colonies) was added to each sample. The samples were

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The purpose of this study was to determine the extent of adsorption of gentamicin to soil.

Design

Gentamicin sulfate (10,000 µg/ml working standard) was added to 5 gram soil samples to yield the following concentrations: 1000, 500, and 250 µg/ml. Five milliliters of 0.1 M PO_4 buffer pH 8.0 was added to each sample. The samples were mixed on vortex mixer - maximum speed 0 then centrifuged for 15 minutes at 20,000 rpm. The final supernatant was assayed against a standard gentamicin curve prepared in 0.1 M PO_4 buffer. The concentrations of the buffer curve were: 0.64, 0.32, 0.16, 0.08, 0.04, 0.02, 0.01, 0 µg/ml. A reference solution containing 0.05 µg/ml gentamicin was prepared in 0.1 M PO_4 buffer pH 8.0 for use in the assay.

Assay Method

The standard and unknown samples were assayed using a microbiologic cylinder plate procedure. The assay organism was S. epidermidis ATCC 12228. Media 11 (agar-special agar noble) was used for the 6 ml base layer and the 3 ml seed layer. The media was adjusted to pH 8.0 and inoculated with a bulk suspension of S. epidermidis ATCC 12228 (O.D. 0.6). Six stainless steel cylinders were placed on the agar at 60° intervals. Three alternate cups were filled with standard or unknown samples. The remaining cups were filled with reference solution. Three 100 x 20 mm petri dishes were plated per standard or unknown sample.

The plates were incubated at 36°C overnight. The resulting zones of inhibition were measured using a Fisher-Lilly Zone Reader. Means were computed for nine readings per sample. The mean of the reference standard was

was adjusted to 50% of the soil moisture capacity. Five-gram samples were prepared for assay using methods previously described. Samples were assayed for gentamicin at 0, 4, 10, 20, and 31 days following the initiation of the study using the previously described microbiological assay procedure. Non-sterile soil samples only showed evidence of microbial activity during the time span of the study.

Results

The results were as follow:

	Gentamicin - % Recovered	
Day	Non-Sterilized Soil	Presterilized Soil
0	1.83	2.43
4	1.71	1.67
10	1.61	1.92
20	0.88	0.99
31	0.80	0.91

Thirty-one days following the initiation of the study, the non-sterilized and presterilized soil samples contained 43% and 37% respectively of the initial free gentamicin recovered from the samples. The cause of the decrease in the percent free gentamicin recovered was not determined due to the limitations of the study.

Summary

Studies indicated that 98% or more of an applied concentration of gentamicin was immediately adsorbed to soil colloids.

The adsorbed gentamicin was not extractable by routine assay techniques and did not show evidence of antibacterial activity against highly sensitive bacteria. Over a period of 31 days, the percent of free gentamicin recovered from non-sterilized and presterilized soil samples treated with gentamicin was reduced by 43% and 37% respectively.

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