Identification and Quantitative Evaluation of Antibiotic Residues in Chickens Fed on a Spiramycin Diet

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A sensitive chromatographic method is described for the determination of antibiotic residues in tissues of chickens fed with spiramycin. Assays were performed on muscles, liver, and kidneys. At a dose level of 10 grams of spiramycin embonate per ton of food, residues (about 0.1 p.p.m.) were found only in the liver; their presence in the other organs, expressed in spiramycin activity, was therefore lower than 0.01 to 0.02 p.p.m. At a dose level of 100 grams per ton, small amounts of metabolites appeared in all the organs, and their approximate concentration was established. The antibiotic residues were identified as spiramycin, neospiramycin, and polar derivatives of neospiramycin. The metabolic pathways which may be involved in the transformation of spiramycin are discussed.

Spiramycin is an antibiotic belonging to the macrolide group; it has been isolated by Pinnert-Sindico *et al.* (1954) from cultures of *Streptomyces ambofaciens* and its structure (Figure 1) has been established by Paul and Tchelitcheff (1965) and Kuehne and Benson (1965). Interest in the possible use of this substance for growth promotion in chickens has led to an investigation of the metabolic fate of spiramycin in these animals and of the biologically active residues which may be found in tissues following the administration of different dose levels of the antibiotic.

The present study summarizes the data obtained in experiments carried out on muscles, liver, and kidneys either as fresh or cooked tissues and indicates the chromatographic methods elaborated to trace and separate the metabolites of spiramycin at very small concentrations.

MATERIALS AND METHODS

Animals. Two groups of 30 chickens each were used. The first group (55 days old) was maintained since hatching on a dose level of 100 grams of spiramycin embonate per ton of food. This dose level corresponds to 10 times the amount considered for growth promotion. The second group (61 days old) was maintained since hatching on a dose level of 10 grams of spiramycin embonate per ton, which represents the real dose considered for food supplementation.

Ten chickens from each group were given the same food without any addition of antibiotics. These animals were used as controls in the chromatographic experiments.

Antibiotic. Spiramycin was used in the form of its embonate—i.e., the salt of 2,2′-dihydroxy-1,1′-dinaphthylmethane-3,3′-dicarboxylic acid.

Extraction Procedure. Analysis was carried out on breast and leg muscles, liver, and kidneys. Half of the birds from each of the treated groups and from each of the control groups were used for the experiments on fresh tissues. The remaining birds were cooked and the analyses were carried out after cooking.

In the first group of birds, tissues were removed and frozen with liquid nitrogen immediately after killing the chickens; they were then freeze-dried and stored as a dry powder at −20° C. until extraction. In the second group, the birds were treated in such a way as to reproduce, as nearly as possible, the normal consumer's conditions. They were stored for 36 hours in a refrigerator at +4° C. and roasted in an oven without addition of fat for 90 minutes; liver and kidneys were treated separately for 25 minutes. Muscles were then removed from the carcass as completely as possible, and all the tissues were freeze-dried and stored at −20° C. until extraction.

Each tissue powder was homogenized with a Teflon blender using the first extraction solvent (benzene). Systematic extractions were then performed with analytical grade solvents in the following order: benzene, methylene chloride, methanol, and water. For 5 grams of dry powder, two 50-ml. portions of each solvent were used; temperatures of 40° to 55° C. do not improve the extraction. The extracts were evaporated to dryness under reduced pressure in a rotating evaporator or freeze-dried in the case of water and subjected to chromatography.

Chromatographic Methods. Crude extracts were too impure for immediate thin-layer chromatography (TLC). Therefore, a purification procedure was elaborated, and the best results were obtained by column chromatography of the extracts on silica gel with a methyl ethyl ketone–methanol solvent system in which the percentage of methanol is gradually increased from 0 to 100%. Notable improvement of the chromatographic separation could be obtained when the silica gel was impregnated with sodium carbonate or bicarbonate. Each fraction of the column eluate was then subjected to thin-layer chromatography after evaporation of the solvent; such combination of two successive fractionation procedures is equivalent to a bidimensional chromatography with the advantage that a single plate may be used for many samples.

For liver and kidney extracts, thin-layer chromatography on silica gel gave satisfactory results after this preliminary purification; for muscle extracts, however, as well as for the separation of the most polar metabolites, special plates were necessary. An applicator fitted with a special mixing chamber was used to coat plates with a mixture of silica gel and cellulose powder so as to have 100% cellulose at the beginning of the chromatograph and 100% silica gel at its end; a continuously varying gradient of the two adsorbents was established between

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these limits. Such a system enables the solvent to elute very polar substances from the loading spot situated in the moderately adsorbing cellulose zone into the strongly adsorbing silica gel area where they are separated. Table I shows that a great gain in sensitivity is obtained in this way. The chromatographs were developed by bioautography with *Bacillus subtilis* ATCC 9341 as sensitive organism and whenever possible by an anisaldehyde spray as indicated later. *Bacillus subtilis* is one of the most sensitive organisms between nonpathogenic germs.

Analytical Procedure. Extracts obtained from each tissue were investigated by means of these two successive chromatographies. A typical experiment is described below.

Thirty grams of silica gel G (Merck) were intimately mixed with a 2% aqueous bicarbonate solution which must be added carefully to the solid to avoid agglomeration of the particles. The excess liquid was removed and the silica gel activated at 130° C. during 30 minutes and cooled in a desiccator. Methyl ethyl ketone was used to prepare a 15 × 0.8 cm. column with this adsorbent.

The whole extract obtained by means of the various solvents mentioned above from 5 grams of dry tissue powder—i.e., about 20 grams of fresh tissue—was stirred in a small cup with about 0.3 gram of untreated dry silica gel in the presence of about 0.5 ml. of methanol. The almost dry powder obtained in such a way was transferred to the top of the column. Twenty to 40 ml. of each of the following solvent systems were then passed slowly through the column: pure methyl ethyl ketone, methyl ethyl ketone–methanol (v./v.): 95 to 5, 90 to 10, 80 to 20, 50 to 50 pure methanol. Ten-milliliter fractions were collected, and the solvent was evaporated. The residue of each fraction was then studied by TLC. Liver and kidney extracts were assayed mainly on usual silica gel plates; for the separation of very polar substances as well as for eluates of muscle extracts which were frequently contaminated with a yellow waxy inactive substance, special thin-layer plates were prepared. One of the compartments of a Dessaga gradient TLC applicator (Roucaire, Paris, France) was filled with a suspension of 30 grams of cellulose G (Merck) in 60 ml. of 2% aqueous sodium carbonate, the other with the suspension of 30 grams of silica gel G (Merck) in 60 ml. of 2% aqueous sodium carbonate. The suspensions were introduced into the mixing chamber, homogenized to obtain a gradient, and immediately spread over the plates. The eluates were spotted on the cellulose-rich side of the plate. The chromatographs were always developed with a methyl ethyl ketone–methanol (85 to 15, v./v.) mixture.

Table I. Minimum Amounts of Spiramycin Detected on Bioautographs of *B. subtilis* with Various Adsorbents

Type of TLC	Adsorbent	Sensitivity
Plate	Silica gel G (Merck)	~0.5 μg.
Plate	Silica gel G (Merck), impregnated with a 2% sodium carbonate solution	~0.1 μg.
Film	Silica gel (K 301 V Kodak), impregnated with a 2% sodium carbonate solution	~0.05 μg.
Plate	Gradient of cellulose G (Merck)–silica gel G (Merck) impregnated with a 2% sodium carbonate solution	0.01–0.03 μg.

Bioautographs were obtained on agar plates, as described by Lederer (1960), with *B. subtilis* ATCC 9341 as the sensitive organism; a 3-hour period was selected for diffusion of the chromatographic spots into the agar, and incubation was carried out for 16 hours at 27° C.

Spiramycin and many of its derivatives, if present at a sufficient concentration, gave blue spots when sprayed with anisaldehyde reagent in sulfuric acid (Stahl and Kaltenbach, 1961) and heated to 140° C. for 3 to 5 minutes for color development. Plates were examined under ultraviolet light as a routine control.

Qualitative and Quantitative Evaluation of Biologically Active Substances. The whole analytical procedure was always run simultaneously on a tissue extract and the corresponding control sample prepared from animals fed on a spiramycin-free diet. The comparison of chromatographs obtained in such a way made it easy to localize the antibiotic or its transformation products; therefore, by means of synthetic references qualitative identification was possible, and by use of several chromatographic systems the identity of the metabolites could be ascertained.

For quantitative determinations of biologically active spots, a first chromatograph gives an approximate value of the antibiotic activity by comparison with spots of increasing amounts of spiramycin or of an identified metabolite sample.

A second—if necessary, a third and fourth—chromatograph was then developed in which the assay sample was compared with spiramycin-free tissue extracts to which known amounts of the reference antibiotic had been added at concentrations very close to the value found in the first chromatograph. When these known and unknown samples finally gave inhibition spots of the same size, the amount of antibiotic present in the tissue extract could be calculated.

The minimum amount of spiramycin derivatives which may be determined by this method as established in control experiments are the following: spiramycin, 0.01 to 0.02 p.p.m.; neospiramycin, approximately 0.02 p.p.m.; forocidine, approximately 0.04 p.p.m.

RESULTS

A complete study of muscles, liver, and kidneys of chickens was undertaken by the methods described above. Controls were always carried out at the same time with tissues from animals fed on a spiramycin-free diet. Once the metabolites had been identified, they were added to antibiotic-free tissues and run through the whole analytical procedure to ascertain their recovery quantitatively.

Identification of Residues. No antibiotic substances were found in tissues from control animals, so that any detected inhibition area on bioautographs can be ascribed to spiramycin or its transformation compounds.

Among the residues which may be present in an extract, three kinds of substances, very well differentiated by their polarity, could be found; the least polar substance is spiramycin, at intermediate polarity neospiramycin was detected, and the most polar group seemed to be mainly

due to conjugate or to bound forms of neospiramycin.

After column chromatography, the distribution of these compounds over the 10-ml. eluate fractions depended on their concentration; they will be found in many fractions if they are present at concentrations higher than 0.25 p.p.m. when the experiments were carried out on the extract of 5 grams of dry tissue powder. At lower concentrations, when 20 ml. of each solvent were used, spiramycin was eluted in fractions 3 to 8, neospiramycin in fractions 5 to 10, and polar derivatives in fractions 6 to 11.

The identity of spiramycin and neospiramycin could be established on thin-layer plates by comparison of their R_f with authentic samples of these substances as indicated in Table II. More difficult was the problem of the most polar substances which appeared in liver tissue extracts as a single spot at the point where they were loaded in many chromatographic systems; use of very polar solvents resulted in a continuous line stretching all over the plate. Resolution, however, was obtained by use of the above described cellulose–silica gel gradient plates, and in solvent systems V and VI (Table II) two spots were observed.

Bidimensional chromatography ascertained the homogeneity of these two compounds. Preparative TLC on silica gel in solvent III was used to get small amounts of these polar substances. After elution of the silica gel with methanol, the two compounds were obtained free from any contamination of spiramycin and neospiramycin. Treatment at 50° to 60° C. with organic solvents—methyl ethyl ketone, for instance—or storage for a longer period at room temperature resulted in their partial transformation into neospiramycin. Spiramycin was never found. These observations suggest that these rather unstable compounds are conjugated or bound forms of neospiramycin. Glycuronidase or sulfatase did not affect the two metabolites.

Quantitative Results. The amounts of the various residues found in tissue extracts estimated by comparison with spiramycin-free tissue extracts to which known quantities of antibiotic reference substances had been added are indicated in Table III. For spiramycin and neospiramycin such references were available; however, this was not the case for the polar derivatives, and therefore only their presence or absence (+ or −) is indicated. However, it is possible to state that the inhibition area attributed to these polar substances on *B. subtilis* autobiographs was in each case smaller than the sum of the inhibition zones due to spiramycin and neospiramycin together.

DISCUSSION

The present study evaluates for the first time by a very sensitive method the amount of each antibiotic residue

Table II. R_f Values of Spiramycin Residues in Some Solvent Systems

Type of TLC	Adsorbent	Solvent System, Vol.	Solvent System	R_f, Spiramycin	R_f, Neospiramycin	R_f, Polar Metabolites
Plate	Silica gel G Merck	Ethylene dichloride–MeOH, 70:30	I	0.30	0.10	0
Plate	Silica gel G Merck	Toluene–MeOH, 50:50	II	0.50	0.15	0
Plate	Silica gel	Acetonitrile–dioxane–MeOH, 30:20:50	III	0.75	0.35	0
Film	Silica gel K 301 V Kodak impregnated with 2% Na_2CO_3	MEK–MeOH, 95:5	IV	0.85	0.55	0 to 0.1
Gradient plate	Silica gel G Merck impregnated with 2% Na_2CO_3	MEK–MeOH, 75:25	V	0.9	0.7	0.40 and 0.25
Gradient plate	Silica gel G Merck impregnated with 2% Na_2CO_3	MEK–H_2O–MeOH, 80:2:20	VI	0.8	0.55	0.30 and 0.15

MEK = methyl ethyl ketone: MeOH = methanol.
R_f's are reproducible at ±8%.

Table III. Quantitative Evaluation of Antibiotic Residues in Tissues

Tissue		Dose Level, Grams/Ton	Spiramycin	Neospiramycin	Polar Derivatives
Muscles	Fresh	100	0.01–0.015 p.p.m.	<0.01 p.p.m.	−
	Cooked	100	<0.01 p.p.m.	<0.01 p.p.m.	−
Muscles	Fresh	10	Unidentified	Unidentified	−
	Cooked	10	Unidentified	Unidentified	−
Liver	Fresh	100	1–1.3 p.p.m.	0.8–1 p.p.m.	+
	Cooked	100	0.8–1 p.p.m.	~0.6 p.p.m.	+
Liver	Fresh	10	~0.12 p.p.m.	~0.07 p.p.m.	+
	Cooked	10	~0.07 p.p.m.	~0.05 p.p.m.	+
Kidneys	Fresh	100	0.12–0.15 p.p.m.	0.1–0.12 p.p.m.	+
	Cooked	100	0.05–0.06 p.p.m.	~0.02 p.p.m.	+
Kidneys	Fresh	10	~0.02 p.p.m.	0.01–0.02 p.p.m.	−
	Cooked	10	<0.01 p.p.m.	Unidentified	−

Data expressed as part per million of fresh tissue.
Dose levels expressed as grams of antibiotic per ton of food.

present in chickens fed for 2 months after hatching on a spiramycin diet. In this investigation, the chickens were fed with a spiramycin-supplemented food until the day on which they were killed, so that maximum residue values were obtained, since a spiramycin-free diet as normally planned during the period preceding death would cause a decrease in the tissue residues.

In the chickens fed at the dose level of 10 grams of spiramycin embonate per ton of food, very small amounts of metabolites were found in the liver, but muscles and kidneys were practically free of them. If one considers the sensitivity of the method, these results mean that the concentrations of antibiotics, expressed in spiramycin activity which may be present in these tissues, are lower than 0.01 to 0.02 p.p.m.

The data provided by the group of chickens treated with ten times the dose considered for growth promotion—i.e., with 100 grams of spiramycin embonate per ton of food—appeared to be most interesting, because at this level, small amounts of metabolites were present in almost all the tissues, which makes a quantitative survey of the residues possible; muscles still had the lowest concentration of metabolites, since the inhibition noted with extracts of these tissues on bioautographs corresponded almost to the limit of sensitivity of the analytical procedure. Of the three tissues investigated, liver had the highest concentration; there was no evidence of qualitative differences of residues between organs.

The exposure of tissues to heat as occurs during the cooking of the chickens decreased the antibiotic activity of the residues in each case to a notable extent but did not destroy it completely.

The quantitative results were obtained as the average values of several experiments. Controls were always carried out to ascertain the completeness of the extraction of the tissues and the quantitative recovery during chromatographic purifications. As to the accuracy of the whole analytical procedure, the figures were calculated as a result of the visual comparison of bioautograph inhibition areas obtained after two chromatographic fractionations; therefore, a notable margin of error must be taken into account.

Spiramycin and neospiramycin are mixtures of the hydroxy, acetoxy, and propionyloxy derivatives designated as spiramycin or neospiramycin I, II, and III (Figure 1). No effort was attempted to separate these compounds for quantitative evaluation as such a fraction, possible in a methyl ethyl ketone–water (50 to 50, v./v.) solvent system, would split the already weak amounts of antibiotics into three spots; furthermore, experiments in which only one constituent was administered did not show any significant difference.

The identity of the more polar spots noted on chromatoplates of liver tissue extracts could not be unambiguously demonstrated; however, their partial transformation into neospiramycin was confirmed in many experiments in which spiramycin remains stable, and therefore the hypothesis of bound or conjugated forms was considered the most probable explanation. The infinitesimal concentrations of these compounds make it impossible to determine by means of a color test the structure of the nonantibiotic part of these conjugates, and enzymatic experiments could only exclude the presence of glucuronates or sulfates.

All the previously discussed metabolites were found through their antibiotic properties. Spiramycins may be easily revealed on chromatographs by several color tests. One of the most sensitive uses a sulfuric anisaldehyde spray (Stahl and Kaltenbach, 1961); the cycles obtained by the splitting of the ether linkages of spiramycin also can be revealed by such methods. However, the limit of sensitivity of this kind of reactions lies between 0.1 and 0.5 μg. in the best conditions—that is to say, when pure samples of controls are chromatographed.

The residue levels found in the tissues of chickens during these investigations were much too low for revelation of the metabolites by the use of color tests. However, during experiments performed with much higher doses of spiramycin (Jolles and Terlain, 1966), especially when chickens

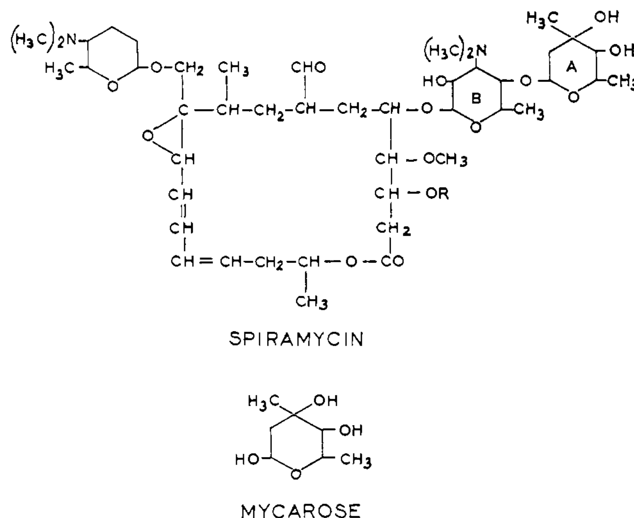

Figure 1. Formulas for the spiramycins and their derivatives (Paul and Tchelitcheff, 1965)

Spiramycin is a mixture of three components:

Spiramycin I.	—R = —H	(63 ± 10%)
II.	—R = $—OCCH_3$	(24 ± 5%)
III.	—R = $—OCCH_2CH_3$	(13 ± 5%)

For the corresponding neospiramycins, A = —H

are killed 24 hours after administration of a single dose of 500 mg. per kg. of spiramycin, sufficient amounts of metabolites were present in the different organs so that mycarose could be found by the anisaldehyde spray as well as spiramycin, neospiramycin, and two polar conjugates in the case of liver extracts. Even at that high dose, no other metabolite was identified.

The main degradation process of spiramycin which has been noted by metabolite identification was therefore the formation of neospiramycin through hydrolysis of the mycarose residue. Neospiramycin in the free form was present in tissues at lower concentrations than spiramycin itself, but appeared capable of conjugation or binding, thus giving rise to polar derivatives which were rather unstable and could be partially retransformed into neospiramycin.

Metabolic pathways other than hydrolysis and conjugation have been studied, mainly in rats (Jolles and Terlain, 1966). No saponification of the acyl group of spiramycin II and III (Figure 1) was found; however, in various tissues, mainly in liver, a strong esterase activity was noted that specifically hydrolyzed the acyl groups of spiramycins chemically acylated on cycle A or B. Some evidence for demethylation of spiramycin could be found in vitro by rat liver homogenates under the conditions used for the erythromycins (Mao, 1965). In vivo in chickens, it was not possible to reveal any metabolite which could be due either to saponification and acylation or to demethylation. Finally, *N*-oxidation could also be excluded by comparison with a synthetic control sample of spiramycin, di-*N*-oxide (Adamski *et al.*, 1966).

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