enantiomers and racemate, and also those of acephate, except that the former was 2-fold more potent than the latter (Table I). This is different from housefly data. The present study clarified the biological activity of both enantiomers of methamidophos and acephate, which were first resolved. Further experiments on metabolic activity using stereochemistry are now being done.

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[^{14}C]Virginiamycin Residues in Eggs

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Laying hens were fed ab libitum a diet supplemented at 40, 10, and 0 mg/kg with [^{14}C]virginiamycin, to determine whether or not virginiamycin or its metabolites were transferred to the eggs. About 0.05% of the ingested ^{14}C dose was recovered in the eggs. Radiolabeled residues expressed in terms of virginiamycin equivalents were found to be 5.1 ng/g in the albumen and 31.8 ng/g in the yolk from hens fed a 10 mg/kg diet. No antibiotic activity could be detected in the eggs. Tentative identifications showed that in the albumen about 17% of the ^{14}C behaved chromatographically like virginiamycin and 18% was associated to the ovalbumin. In the yolk 31% of the ^{14}C was associated to proteins, 58% to fatty acids, and 4% to unsaponifiable matter.

Virginiamycin is an antibiotic used in poultry husbandry at levels of 7.5–40 mg/kg to improve poultry performance. Virginiamycin-supplemented diet stimulates growth and improves feed efficiency in broilers. It increases egg production in laying hens (Keppens et al., 1981; Miles et al., 1985).

Numerous studies have shown that detectable levels of antimicrobial residues can occur in eggs following the administration to hens of antibiotics at therapeutic levels (Petz, 1984). However, residues could not be detected in eggs by microbiological methods, after the administration of antibiotics at growth-promoter levels (Katz et al., 1974).

Virginiamycin seems to be absorbed in the rat, since 17% of the administered ^{3}H disappeared from the ligated stomach after an oral dose of virginiamycin [^{3}H] factor M_1 (Roberfroid and Dumont, 1972) and since about 88% of an oral dose of [^{14}C]virginiamycin was recovered in feces of rats and cattle (Gottschall et al., 1987). Virginiamycin seems to be less absorbed in poultry, since the plasma concentration of radioactive residues was found to be 0.04 μg/g in broilers given for 12 days a diet supplemented at 44 ppm (activity) (Miller, J. A., personal communication). Microbiological assays of eggs from hens fed a diet containing 40 ppm (activity) virginiamycin for 6 months showed no detectable antibiotic residues, the detection limits being 20 ng/g in the albumen and 50 ng/g in the yolk (Di Cuollo, 1980).

Since in European countries virginiamycin is currently being approved for use in laying hens at 10–40 ppm (activity) inclusion level, the objective of the present study was to determine the amount and the nature of residual radioactive material in eggs from hens fed diets supplemented with 10 and 40 mg/kg of [^{14}C]virginiamycin. According to Smith Kline Corp., which provided us with the drug, and due to improvements in the synthesis procedure, the microbiological activity of the drug we used was 200%. This means that the 10 and 40 mg (weight) of drug we added per kilogram of diet corresponded to commercial feedstuffs containing 20 and 80 ppm (activity) of virginiamycin, respectively.

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MATERIALS AND METHODS

[^{14}C]Virginiamycin. Generally labeled [^{14}C]virginiamycin was provided by Smith Kline Corp. It was prepared by fermentation from [^{14}C]acetate and different ^{14}C amino acid precursors used simultaneously (Gottschall et al., 1987). The radiochemical purity of the drug was checked by thin-layer chromatography (TLC), using two solvent systems, followed by radio-TLC scanning. Two radioactive areas were detected, which corresponded to factors M (66–68%) and S (32–34%) of virginiamycin. This preparation of virginiamycin was assayed vs the original activity standard and was twice as active as the original standard, for an equivalent sample weight (Gottschall et al., 1987).

Preparation of Experimental Feed. Usual precautionary handling procedures were followed each time radioactive material was used. [^{14}C]Virginiamycin, 30.4 kBq/mg, was dissolved in ethanol, and a known volume was adsorbed onto the feed pellets of a standard laying hen diet, so that the diets contained either 10 mg/kg of virginiamycin and 304 Bq/g or 40 mg/kg and 1215 Bq/g. Due to the 200% microbiological activity of the product, these actual concentrations (weight basis) corresponded to 20 and 80 g/ton supplemented feed, respectively, ac-

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tivity basis. Control feed were treated with a similar volume of ethanol. In order to remove the solvent, the containers were allowed to stand at room temperature for about 24 h.

Hen Treatment and Egg Collection. Warren Isa Brown hens, 18 weeks of age, were housed in individual cages and allowed to acclimatized to housing and diet for 4 weeks. The birds were adapted progressively to a 16-h light exposure and attributed at random to three groups. One group was given the 10 mg/kg supplemented feed for 15 days (six hens), the second group the 40 mg/kg supplemented feed for 17 days (six hens), and the third group the ethanol-treated feed (control group, three hens). Feeds were given ad libitum, and the weekly intake of each hen was recorded. Eggs were collected every day during a 40-day period. To avoid contamination of the albumen with radioactive material from the shell, eggs were allowed to cool at room temperature before being washed carefully with water at the same temperature. The eggs of three hens of each experimental group were stored at 4 °C. The eggs of the three other hens were processed as follows the day of laying. Just after washing, yolk and albumen were separated and their weights determined. After blending, aliquots of each were counted for total radioactivity. The remaining material was frozen for later analysis.

Microbiological Assays of Albumen and Yolk. Extraction was made using 60 mL of ethyl acetate, which was added to 10 g of albumen or to 5 g of yolk, and the mixture was then gently stirred for 5 min. After centrifugation, the supernatant was dried under vacuum and dissolved in 0.4 mL of ethanol for the albumen. The yolk-dried extract was dissolved in 10 mL of acetonitrile and extracted twice with 20 mL of isooctane. The acetonitrile phase was dried under vacuum, and the residue was dissolved in 0.4 mL of ethanol. The extracts (80 μL) were applied to paper disks. After drying, the disks were laid on agar antibiotic medium 1 (Difco) inoculated with *Sarcina lutea* ATCC 9341. The diameters of the inhibition zones obtained after diffusion (60 min, 20 °C) and incubation (24 h, 30 °C) were compared with a standard responce curve established from control eggs spiked with known amounts of virginiamycin. This addition was made with virginiamycin dissolved in ethanol and then diluted in water, so that 45 μL of water and 5 μL of ethanol were added to 10 g of egg. The detection limits were 20 ng/g in albumen and 50 ng/g in yolk.

^{14}C Analysis. Five aliquots of 2 g of albumen and five aliquots of 0.4 g of yolk were counted by liquid scintillation counting (LSC) on a TriCarb 4000 (Packard), for 30 min, after addition of 10 mL of a fluor mixture (Lumagel, Kontron, France). The background was determined every day by the same procedure on control eggs. The detection limit of the counting procedure was determined with control eggs spiked with [^{14}C]virginiamycin and was found to be 28 mBq/g of albumen and 13 mBq/g of yolk (p = 0.05 in the Student's t-test).

Thin-Layer Chromatography. Silica gel TLC plates (60, Merck) were activated (110 °C, 1 h) and spotted with the ethyl acetate extract prepared from the whole albumen of one egg (40 g approximately). Albumen extracts from hens fed the 40 mg/kg diet were spotted in parallel with the extract of control egg spiked with [^{14}C]virginiamycin. The plates were developed with dichloromethane–methanol (95:6.5, v/v) and then observed under UV light (254 nm). Adjacent strips were delineated between the deposit line and the solvent front, and the silica was scrapped off and collected onto a filter paper placed on a funnel. Two-milliliter portions of water were added and then twice 10 mL of dichloromethane. The radioactivity of each area was determined by LSC of the corresponding dichloromethane extract after concentration under vacuum.

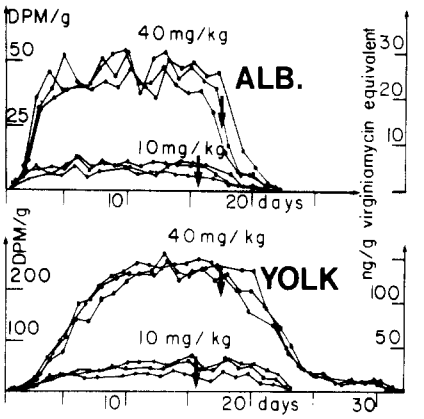

Figure 1. Recovery of ^{14}C in albumen (ALB) and yolk from eggs of hens fed [^{14}C]virginiamycin. Each point represents an average from LSC counting of five aliquots from one egg. Three hens fed each diet were monitored. Arrows indicate withdrawal days.

Exclusion Chromatography. Albumen was diluted to 1/8 with 0.2 M NaCl, and 3 mL of this dilution was chromatographed on a Sephadex G-50 column, 1.6 × 40 cm, with a pH 7.5 buffer (Tris 50 mM, NaCl 50 mM) at a 30 mL/h flow rate. Fractions (1.5 mL) were collected, then passed through a UV monitor at 280 nm to determine the protein content, and finally counted for radioactivity by LSC. When necessary, these fractions were pooled and then freeze-dried before counting.

Ovalbumin Crystallization. Globulins were removed from albumen by precipitation with ammonium sulfate (50% saturated). Crystallized ovalbumin was then isolated from the supernatant by three sequential precipitations with saturated ammonium sulfate at pH 4.6 (Sorensen and Hoyrup, 1917). Freeze-dried ovalbumin (200 mg) was dissolved in 2 mL of water and counted by LSC.

Yolk Lipid Analysis. Total lipids were extracted from yolk by the method of Folch et al. (1957). Aliquots of 100, 200, and 500 mg of the extracted lipids were counted by LSC. Residual proteins were dialyzed and freeze-dried before LSC counting.

Saponification was carried out on 5 g of yolk following the addition of 50 mL of ethanolic potassium hydroxide (10%, w/v). The mixture was blended with a Polytron mixer for 1 min and then heated for 3 h at 90 °C. After cooling, 50 mL of water was added, and the unsaponifiable matter was extracted with hexane (50 mL). The aqueous phase was acifidied to pH 1 with concentrated HCl, and the fatty acids were extracted with hexane (50 mL). Both hexane fractions were dried under vacuum, and the residue was dissolved in toluene and then counted by LSC.

Pharmacokinetic Analysis. Data were analyzed with a minicomputer (Mini 6, CII) using a program for nonlinear regression analysis (Yamakoa et al., 1981), and the most suitable regression equation was estimated.

RESULTS

The administration of the supplemented feed had no significant effect on daily food intake, egg production, albumen, or yolk weight during the relatively short experimental period.

Recovery of ^{14}C in Albumen and Yolk of Eggs. About 0.05% of the ingested ^{14}C was excreted in the eggs. The incorporation and elimination of ^{14}C in the albumen and yolk of eggs from three hens given the 40 mg/kg diet and three hens given the 10 mg/kg diet are shown in Figure 1. The data obtained with the 40 mg/kg diet show that the radioactivity reaches a steady-state level after 3–4

Table I. Recovery of ^{14}C and Virginiamycin Equivalents in Eggs from Hens Given [^{14}C]Virginiamycin

[^{14}C]virginiamycin concn in diet, mg/kg	recovered ^{14}C, % of dose	[^{14}C]virginiamycin equiv, ng/g	
		albumen	yolk
10	0.052	5.1 ± 1.1[a]	31.8 ± 5.8
40	0.054	23.2 ± 4.3	124.2 ± 15.0

[a] Mean ± standard deviation from 12 eggs. For each egg, five aliquots were counted for 30 min.

days of [^{14}C]virginiamycin administration in the albumen and 10 days in the yolk. The increase of ^{14}C in the albumen was exponential [$y = 0.97e^{1.2t}$; $r = 0.99$] while it was linear in the yolk [$y = 27.4t - 38.7$; $r = 0.98$], where y is the dpm/g of egg at time t (days) following the start of drug administration and r is the correlation coefficient. The decrease of ^{14}C after the withdrawal of dietary virginiamycin was exponential in the albumen [$y = 38.3e^{-0.7t}$; $r = 0.98$] and in the yolk [$y = 364\ e^{-0.28t}$; $r = 0.97$], where t is the time after drug withdrawal. The cpm/g values of albumen and yolk from hens given the 40 mg/kg diet were not different from those of control eggs 4 days (albumen) and 14 days (yolk) after withdrawal. The mean ^{14}C residues in eggs at the plateau of equilibrium measured on two consecutive days in the eggs laid by the six hens of the two supplemented groups are shown in Table I. The amounts of virginiamycin equivalent in eggs are shown in the same table and were found to be 5.1 μg/g in the albumen and 31.8 μg/g in the yolk from hens fed the 10 mg/kg diet. The data indicate that the residual level in eggs is proportional to the ingested dose and that the specific activity of the dry albumen is similar to that of the dry yolk. Due to the very low residue levels in eggs, the tentative identifications of the radioactivity were all made on eggs from hens given the 40 mg/kg regimen.

Nature of ^{14}C in Albumen. Table I shows that 23 ng/g [^{14}C]virginiamycin equivalent was found in albumen from the 40 mg/kg supplemented hens. Although the detection limit of the microbiological assay was 20 ng/g of standard virginiamycin, no inhibition zone was found when albumen from eggs collected on days 5, 9, and 12 after the start of the experimental diet were assayed microbiologically.

The mean percentage of ^{14}C extractable by ethyl acetate from albumen was found to be 34 ± 14 in experimental eggs ($N = 40$) vs 74 ± 11 in control eggs spiked with 50 ng/g standard [^{14}C]virginiamycin ($N = 38$). This last yield was not improved either by changing the extraction pH, increasing the ionic strength (until 0.6 M), or adding 4 μg/g of unlabeled virginiamycin in the albumen as a carrier, before the extraction procedure. The radio-TLC analysis of albumen extract (Figure 2) shows two radioactive peaks of R_f corresponding to virginiamycin factors M and S. The ^{14}C contents of these two fractions were 47 and 7%, respectively, of the albumen extract (mean of three assays). However, the identity of these fractions could not be positively ascertained due to the very low quantities available.

The exclusion chromatography of albumen from control eggs spiked with [^{14}C]virginiamycin shows two radioactive peaks (Figure 3). The major one was eluted with a retention volume corresponding to pure virginiamycin, and the minor one was eluted together with the ovalbumin. When different amounts of virginiamycin and albumen were chromatographed together, a significant correlation coefficient of 0.997 was found between the amount of albumen and the ^{14}C eluted together with ovalbumin. This phenomenon of coelution of virginiamycin together with ovalbumin seems to reflect some interaction between the

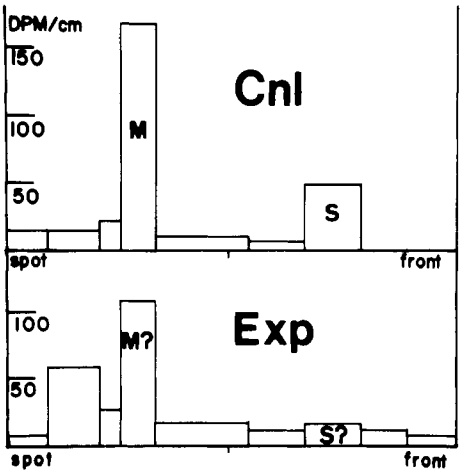

Figure 2. Radio-TLC of ethyl acetate extract of albumen from hen fed [^{14}C]virginiamycin (Exp) and extract of a control albumen spiked with [^{14}C]virginiamycin (Cnl). The experimental conditions are given under the Experimental Section.

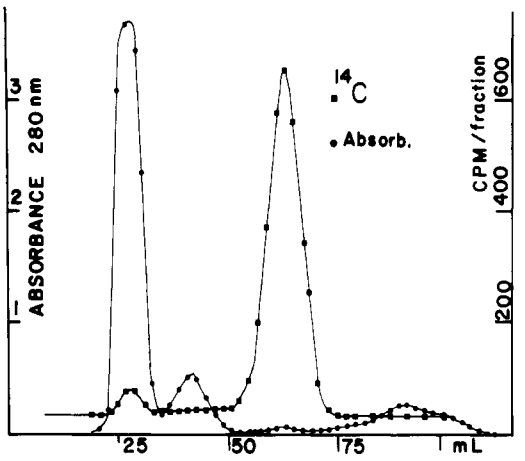

Figure 3. Chromatograph of albumen, from control egg spiked with [^{14}C]virginiamycin, on a Sephadex G 50 column.

molecules and may explain the partial extraction coefficient (74%) of the drug from spiked control albumen. The fractions collected after chromatography of albumen from experimental eggs did not contain enough ^{14}C to be measured by LSC.

A specific activity of 1.7 Bq/g of protein was found in ovalbumin crystallized from experimental eggs. The ovalbumin ^{14}C thus contributed to 17.6 ± 1.3% of the albumen radioactive content (three eggs were tested). In a control experiment, the ovalbumin crystallized from control eggs spiked previously with 50 ng/g of [^{14}C]virginiamycin contained no detectable ^{14}C.

Nature of ^{14}C in Yolk. Table I shows that 124 ng/g [^{14}C]virginiamycin equivalent was found in yolk from the 40 mg/kg supplemented hens. Although the detection limit of the microbiological assay was 50 ng/g pure virginiamycin, no inhibition zone was found when yolk from eggs collected on days 5, 9, and 12 after the start of the experimental diet were assayed microbiologically.

The mean percentage of ^{14}C extractable from yolk, using ethyl acetate followed by acetonitrile–isooctane partition, was found to be 5.0 ± 1.7 in experimental eggs ($N = 12$), vs 78 ± 11 in control eggs spiked with 50 ng/g [^{14}C]virginiamycin ($N = 6$). The extracted ^{14}C from experimental yolks could not be identified. Total lipids extracted from experimental eggs contained 65.3 ± 12.7% of the yolk ^{14}C ($N = 9$). Fatty acids and unsaponifiable matter contributed respectively to 57.9 ± 13.3% and 3.7 ± 1.4% of the

Table II. Distribution of ^{14}C in Albumen and Yolk Constituents from Hens Fed [^{14}C]Virginiamycin

constituent	total	extractable[a]	proteins	lipids
albumen	28.1[b]	9.6	7.6[c]	
yolk	71.9	3.6	22.0	44.3

[a] Extractable in the same conditions as standard virginiamycin in control eggs. [b] All the data are mean percentage of total ^{14}C in one egg, the whole egg being considered as 100%. [c] Extrapolated from ^{14}C in ovalbumin.

experimental yolks radioactive content (mean of 14 and 9 saponifications, respectively). The specific activity of the unsaponifiable matter (14.5 ± 2.1 Bq/g) was about twice that of fatty acids (6.6 ± 0.8 Bq/g, $N = 4$). However, no ^{14}C was found in the fatty acids nor in the unsaponifiable fractions prepared under similar conditions from control yolks spiked with 50 ng/g [^{14}C]virginiamycin. A specific activity of 7.2 ± 0.5 Bq/g was found in proteins obtained after yolk extraction with isooctane, which thus contributed to 30.6 ± 2.1% of the experimental yolk radioactive content.

Table II shows that the origin of 61% of the albumen ^{14}C and of 97% of the yolk ^{14}C could be assigned to egg constituents and to extractable matter. About 13% of the egg ^{14}C could not be attributed, mainly located in the albumen.

DISCUSSION

It is important to consider the withdrawal period necessary to clear the residues from the eggs when therapeutic antibiotics are concerned (Löliger, 1978). However, when feed additives like virginiamycin are fed continuously to poultry, the most important parameters to consider are the level and nature of residues in eggs at the plateau of equilibrium. The data show that only 0.05% of the ^{14}C was transferred from the diet to the egg and that about 90% of this radioactivity was not extractable in conditions similar to those retained for free virginiamycin (Table II). This can be compared with tissues of rats, turkeys, and cattle given oral doses of [^{14}C]virginiamycin, where 56–73% of the liver residues cannot be extracted by solvents (Gottschall et al., 1987).

Most of the proteins of the albumen are formed in the magnum during a 3-h time course, about 22 h before the egg is laid (Sturkie, 1965). This explains why the albumen of the eggs laid on day 2 of the experiment contains little ^{14}C. The plateau of equilibrium is reached on day 3 or 4 in the albumen, and it can be assumed that it reflects the evolution of the ^{14}C plasmatic level, with a 24-h time delay. Whether the ^{14}C associated with ovalbumin was due to virginiamycin metabolites linked to the protein by covalent binding or to the incorporation of virginiamycin fragments during the biosynthesis of the albumin could not be investigated further, due to the very low ^{14}C levels.

The linear increase of ^{14}C in the yolk, and the 10-day delay between the start of ^{14}C administration and the plateau of equilibrium, mimics the incorporation kinetics of lipids in yolk measured either by the changes in weight of the ovum (Sturkie, 1965) or by the incorporation kinetics of ealaidic acid in the yolk (Leclercq, 1967). It can be modeled by the successive deposit of yolk layers, with the same specific activity. This phenomenon of a linear increase of drug concentration in the yolk, followed by an exponential decrease after the drug withdrawal, was previously observed in eggs from hens fed kanamycin (Yoshida et al., 1976) and [^{14}C]deoxynivalenol (Prelusky et al., 1987) supplemented diet. In the yolk, the radiolabeled fatty acids may result from the incorporation of virginiamycin fragments during the biosynthesis of lipids. The same explanation applies to the insaponifiable matter, at least when cholesterol is concerned.

On the basis of the proportionality between the dietary dosage and the residue levels measured at the steady state (Table I), one can assume that the concentration of virginiamycin-like residues extractable with ethyl acetate, in eggs from hens fed a 10 mg/kg supplemented diet, might be one-fourth of the level estimated in eggs from hens fed the 40 mg/kg diet. Thus, the maximum possible virginiamycin concentration should be 1.7 ng/g in the albumen and 2.0 ng/g in the yolk, hence yielding a maximum total antibiotic residue of 97 ng of virginiamycin per egg.

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