

Full Length Research Paper

The impact of diflubenzuron (DFB) feeding on glycosaminoglycan and sulfhemoglobin biosynthesis in one day hatched chicks *Gallus domesticus* (L).

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Diflubenzuron (Dimilin), an inhibitor of insect chitin synthesis, was fed to neo-hatched chicks in concentrations of 50 to 2000 ppm in the diet. The activity of the vertebrate hexosamine transferases responsible for glycosa-minoglycan (mucopolysaccharide) formation was monitored by measuring the *in vivo* rate of incorporation of a labeled precursor into hyaluronic acid and chondroitin sulfate of skin. No inhibition of biosynthesis was noted at any concentration; indeed the insecticide appeared to stimulate the synthesis of these compounds. After 3 weeks on the diet, chicks eating 1000 and 2000 ppm diflubenzuron showed obvious signs of cyanosis, and sulfhemoglobin was demonstrated in the blood of chicks eating more than 200 ppm. The amount of sulfhemoglobin appeared to be related to the dietary insecticide content and the highest level seen was 13% of total hemoglobin in the 2000 ppm group after 31 days. These values returned to normal within 3 weeks when diflubenzuron was removed from the diet.

Key words: Pesticides, insect growth regulators, diflubenzuron, chicks, glycosaminoglycan, sulfhemoglobin, *Gallus domesticus*.

INTRODUCTION

Diflubenzuron (Figure 1), is the common name of a new insecticide with the proprietary name Dimilin, and a chemical name using IUPAC nomenclature of 1-(4-chlorophenyl)-3-(2, 6-difluorobenzoyl)-urea. This compound interferes with the biosynthesis and deposition of chitin in the cuticle during the insect molting process (Rouabhi et al., 2006a, 2006b). It is proposed (Ferrell and Verloop, 1976) that the mode of action of diflubenzuron is analogous to that of the fungicidal antibiotic polyoxin-D which inhibits chitin synthesis (Soltani et al., 1995), the enzyme responsible for the incorporation of UDP-N-acetylglucosamine into chitin. Although insect chitin synthetase is notoriously difficult to isolate, the circumstantial evidence implicating diflubenzuron as a direct inhibitor of this enzyme is very strong (Endo et al., 1970; Post and Vincent, 1973; Post et al., 1974). The connecti-

ve tissue glycosaminoglycans of vertebrates are formed by the transfer of glucose derived UDP-N-acetyl-hexosamine into the growing polysaccharide chain (Roden and Schwartz, 1975). The major cartilage constituent, chondroitin sulfate, contains galactosamine, and the more widely distributed hyaluronic acid contains glucosamine. Thus, before large amounts of diflubenzuron are introduced into the biosphere, it should be ascertained whether the biosynthesis of vertebrate's glycosaminoglycan is inhibited in a manner analogous to the inhibition of chitin biosynthesis. This investigation was conducted to determine the effect of diflubenzuron on glycosaminoglycan biosynthesis in chick's tissues.

MATERIAL AND METHODS

Our chemical material is diflubenzuron, a benzoylphenyl urea compound, one of insect growth regulators (IGRs) family. The solubility in acetone is 6.5 g/l at 20°C, but it is poorly soluble in water (8 x 10⁻³ g/l at 25°C and pH 5.6).

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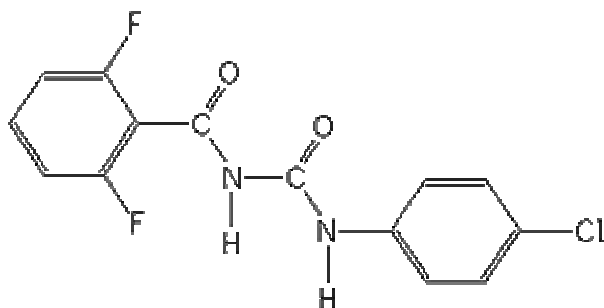


Figure 1. Chemical structure of Diflubenzuron (Rouabhi, 2002).

Pesticide administration

Chick diet from market was ground to a fine powder, using a mill. This powder was then used to prepare a chick diet containing varying concentrations of diflubenzuron. A one-week supply of food was conveniently prepared using the following recipe: 28 g of gelatin were dissolved in 687 ml of hot tap water (60°C) and thoroughly mixed with 672 g of ground powder to provide a stiff paste. When this paste had cooled to about 30°C, 13 ml of a diflubenzuron solution in dimethyl sulfoxide was added (varying concentrations dependent upon the final desired concentration). The food was prepared in large plastic dish pans and pressed into a flat cake about 0.7 cm thick. This was then refrigerated until the gelatin had set, yielding a moist cohesive cake which could be cut with a spatula into small cubes which were stored at 4°C until use. Diflubenzuron was added to final concentrations of 50, 200, 400, 1000, and 2000 ppm (w/w, i.e., µg diflubenzuron/g of dry diet constituents). Birds were fed daily, and unused food was replaced with fresh one.

Experimental design

Diflubenzuron was administered to chicks. Five groups of 10 chicks each were fed the diet containing diflubenzuron at different concentrations. A sixth group of 10 control chicks was fed a diet prepared as above but with no diflubenzuron. The chicks were observed and weighed on alternate days to determine whether obvious signs of toxicity developed. After 30 days on the diet, 5 chicks from each group were injected intraperitoneally with 15 µl of (¹⁴C glucose and killed 8 h later). The animals were defeathered and their entire skins removed and weighed prior to being frozen in liquid nitrogen and pulverized with a stainless-steel crushing device (Walser and Bodenlos, 1954). Hyaluronic acid and chondroitin sulfate were both isolated from the skin. The pulverized tissue was suspended in 0.1 M phosphate buffer, pH 6.5 containing 4.5 mM cysteine-HCl, 5.0 mM disodium EDTA and 0.25 mg/ml twice-crystallized papain, and the mixture was incubated at 50°C for 16 h. The entire digest was then dialyzed at 4°C for 24 h against water prior to differential precipitation of the glycosaminoglycans with acetyl pyridinium chloride (CPC).

Differential precipitation procedures

Polyanions such as hyaluronic acid and chondroitin sulfate form water-insoluble salts with CPC dependent upon the salt concentration. The CP- hyaluronate complex dissociates at concentrations of NaCl above 0.3 M, whereas the CP-chondroitin sulfate complex is stable below 1.0 M NaCl. This phenomenon was originally described by Scott (1960) and a variety of separation schemes has

been based upon it (Antonopoulos et al., 1964; 1961). The procedure used here is the batch procedure which we have previously described (Rokosova-Cmuchalova and Bentley, 1968). The papain digest, after removal of amino acids and salts, was further dialyzed into a final concentration of 0.45 M NaCl. 1% of CPC (Acetyl pyridinium Chloride) was added until all of the chondroitin sulfate precipitated. This precipitate was removed by centrifugation and the supernatant was diluted to 0.15 M NaCl by the addition of 0.05% CPC. Since this concentration is below the critical electrolyte concentration for the hyaluronic acid-CP complex, a precipitate formed which was removed by centrifugation. The precipitated hyaluronic acid and chondroitin sulfate were freed of CPC by dissolving in 60% propanol. Ethanol was added to this solution to a final concentration of 70%, upon which the hyaluronic acid or chondroitin sulfate precipitated, leaving CPC in solution. The precipitated mucopolysaccharides were dissolved in a known volume of water prior to analysis. Aliquots of the aqueous solution were disscintillation spectrometer. Other aliquots were assayed for uranic acid content by the automated procedure of Rosenthal et al. (1976).

Statistical study

All results are shown in means ± standard error. The software used is Minitab 14.20. The tests are: the (*t*) student test and Dunnett method for comparison and of course, variance analysis.

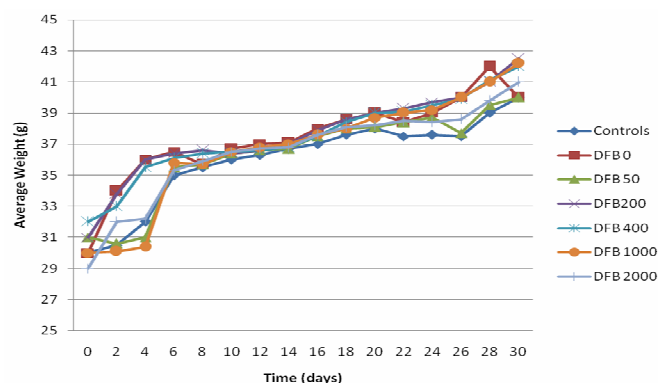


Figure 2. Weight gain of chicks fed diflubenzuron. Animals were fed chick food containing gelatin and diflubenzuron dissolved in DMSO. Food was changed daily. Animals were weighed on alternate days ($p > 0.05$).

RESULTS AND DISCUSSION

The weights of chicks in the six groups receiving the special diet containing from 0 to 2000 ppm of diflubenzuron were compared with those of a seventh group of chicks eating regular chick food, and the results are shown in Figure 2. No major differences in weight gain can be seen for any of the treated animals. All of the animals on the special diet grew slightly better than those kept on regular chick food, and we attribute this to the increased protein content (gelatin), since the average consumption for all chicks was approximately 5-7 g dry weight of food/ chick/day.

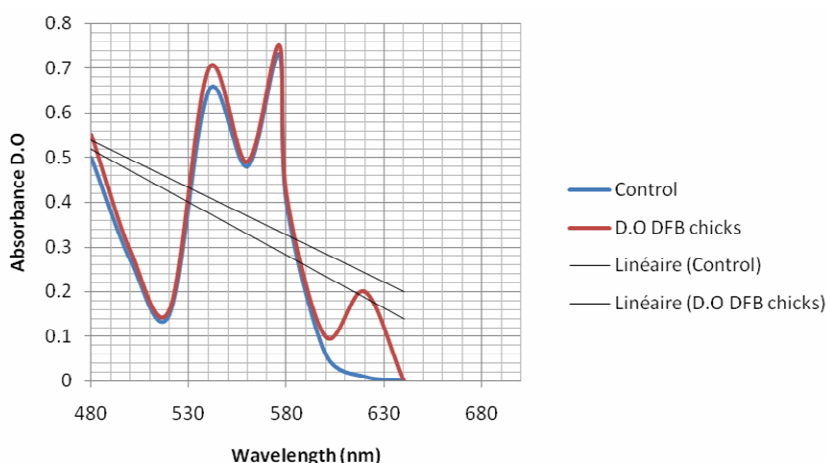


Figure 3. Chick hemoglobin absorption scan. 50 μ l of blood were diluted with 10 ml of 0.015M PO_4 buffer, pH6.6, and clarified by centrifugation. Control blood shows the normal hemoglobin absorption spectrum with maxima at 540 and 576 nm. An additional peak at 619 nm characteristic of sulfhemoglobin is seen in blood from diflubenzuron fed chicks.

Table 1. Hemoglobin analysis of blood from chicks fed diflubenzuron for 30 days (n=3).

Dietary DFB content (ppm)	HGB (g/dL)	SHGB* (g/dL)	SHGB* (%)
DFB** 0	13.40 \pm 0.75	0	0
DFB 50	14.65 \pm 0.43	0	0
DFB 200	14.20 \pm 0.39	0.22 \pm 0.05	1.84 \pm 0.35
DFB 400	14.10 \pm 0.55	0.49 \pm 0.05	3.58 \pm 0.38
DFB 1000	14.53 \pm 0.85	1.07 \pm 0.44	7.19 \pm 2.64
DFB 2000	14.13 \pm 0.67	1.89 \pm 0.70	13.25 \pm 4.49

*SHGB: Sulfhemoglobin. **DFB: Diflubenzuron

Development of sulfhemoglobinemia

After approximately 3 weeks of feeding the chicks receiving the higher dose levels (1000 and 2000 ppm) of diflubenzuron began showing signs of cyanosis. Blood was drawn from the wing vein of control chicks and chicks from the 2000 ppm group and analyzed for the presence of methemoglobin and sulfhemoglobin (Henry, 1967) using a recording spectrophotometer. Figure 3 shows a scan of hemoglobin from the control and from the 2000 ppm group of chicks. Peaks at 540 nm and 576 nm demonstrate hemoglobin and oxyhemoglobin in both samples. A prominent absorption peak at 619 nm, characteristic of sulfhemoglobin, was seen in the experimental group, but no significant absorbance was detected at 630 nm, the absorption maximum of methemoglobin. Methemoglobin, if present, can be completely converted to cyanmethemoglobin by the addition of sodium cyanide, and the absorption peak at 630 nm is abolished. Conversely, cyanide does not affect the absorption of sulfhemoglobin (Henry, 1967). In control blood samples in which methemoglobin had been generated by

the addition of potassium ferricyanide, a peak at 630 nm was observed, and this was completely abolished by the addition of one drop of neutralized 10% sodium cyanide solution to 10 ml of the diluted hemoglobin solution as used in Figure 3. Addition of sodium cyanide in this manner to a hemoglobin solution from the experimental animals left the peak at 619 nm completely unchanged, characteristic of the presence of sulfhemoglobin (Henry, 1967). In order for sulfhemoglobin to be firmly reported as being present, a peak at 617 - 621 nm must be observed after one drop of concentrated NH_4OH has been added (Henry, 1967). The peak at 619 nm in the hemoglobin preparation from the experimental animals was completely stable to the addition of NH_4OH , and we concluded that the administration of diflubenzuron to chicks resulted in the appearance of sulfhemoglobin with undetectable amounts of methemoglobin. At the conclusion of the 30 day feeding study, blood samples were taken from three chicks of each group and analyzed for total hemoglobin, oxyhemoglobin, and sulfhemoglobin by standard procedures as described by Henry (1967). The results are shown in Table 1. Those chicks fed diflu-

Table 2. Glycosaminoglycan analysis of skin from chicks fed diflubenzuron* Concentration of Diflubenzuron.

	Control	400	1000	2000
Chondroitin sulfate				
μg uronic acid/g tissue	47.05 \pm 2.76	70.45 \pm 2.71	75.45 \pm 2.61	82.68 \pm 5.65
dpm**/ μmole uronic acid	2583.8 \pm 277.8	2281.7 \pm 221.6	2356.6 \pm 221.1	2063.2 \pm 241.9
dpm/g tissue	620.5 \pm 81.4	816.2 \pm 83.8	895.8 \pm 76.9	884.1 \pm 125.0
Hyaluronic acid				
μg uronic acid/g tissue	33.05 \pm 6.85	58.42 \pm 4.30	59.62 \pm 4.06	21.47 \pm 1.91
dpm/ μmole uronic acid	2987.0 \pm 425.9	3077.0 \pm 256.3	2710.6 \pm 216.2	2960.4 \pm 341.2
dpm/g tissue	410.2 \pm 90.7	904.2 \pm 69.0	847.7 \pm 103.7	311.2 \pm 27.2

*Results are expressed as mean \pm SEM. Data within a bracket are not significantly different but all data in a bracket are significantly different from data outside that bracket ($P \leq 0.05$). ** dpm: specific activity

benzuron at 50 ppm exhibited no detectable sulfhemoglobin. Animals fed 200 ppm and greater showed a marked increase in the percentage of sulfhemoglobin, and the content appeared to be dose-dependent. After 30 days of eating a diet of 2000 ppm, chicks demonstrated a content of sulfhemoglobin which was approximately equal to 13% of their total hemoglobin. The structure and the occurrence of sulfhemoglobin in the blood are poorly understood. Its occurrence, however, has been noted following the administration of acetanilide, phenacetin, and sulfonamides with levels rarely exceeding 10% of the total hemoglobin (Dubowski, 1964). Since sulfhemoglobin is not reconverted to oxyhemoglobin *in vivo*, its rate of disappearance from the blood is determined by the life span of the erythrocyte (Dubowski, 1964). At the conclusion of the feeding experiments, the test food of the excess animals not used in the biosynthesis studies was replaced with normal laboratory food. Approximately 3 weeks later, all signs of cyanosis had disappeared, and when blood samples were again taken and analyzed, the highest value obtained was 0.14% in the 2000 ppm group.

Hyaluronic acid and chondroitin sulfate biosynthesis

The results are shown in Table 2 and are expressed as means \pm standard errors of the means. Statistical analysis of appropriate groups was performed using an analysis of variance procedure. There was no significant decrease in the specific activity (dpm/ μmole of uranic acid) in chondroitin sulfate or hyaluronic acid isolated from the skin of any of the treated chicks. This indicates that there was no inhibition of biosynthesis and that diflubenzuron does not inhibit the hexosamine transferases responsible for the biosynthesis of these mucopolysaccharides.

Chondroitin sulfate biosynthesis

The total incorporation of label (dpm chondroitin sulfate/g of tissue) increased at all dose levels, which means that

during the 8 h of the *in vivo* labeling experiment each gram of skin incorporated about 50% more label into chondroitin sulfate than did the control skin. Similarly, the results in Table 2 show that the total amount of chondroitin sulfate present in this skin (μg uronic acid/g tissue) was also increased at all dose levels, which means that the increased rate of biosynthesis observed during the acute 8 h period was a continuing process, apparently leading to an increased pool size of chondroitin sulfate in the skin of the treated animals. This increased pool size coupled with the increased rate of label incorporation during the acute experiment can only be interpreted as an increased rate of biosynthesis, since a decreased rate of degradation in the face of a constant rate of synthesis would not give these results. Under these circumstances, we would expect to see an increased amount of uronic acid/g of tissue and a decrease in the dpm/g of tissue.

Hyaluronate synthesis

The data presented in Table 2 for hyaluronic acid can be interpreted in a similar manner to that for chondroitin sulfate. The specific activity was not significantly different in any of the groups. Treatment with the insecticide at 400 and 1000 ppm appeared to stimulate the biosynthesis of hyaluronate. There was, however, a noticeable decrease at the highest dose level of 2000 ppm. It is, of course, possible that the increases seen in the biosynthesis of chondroitin sulfate and hyaluronic acid may be more relative than absolute and could be due to large decreases in the concentration of some other skin component, such as water, collagen, or lipid, which were not measured. At this time, attempts to explain the apparent increase in mucopolysaccharide biosynthesis would therefore be pure speculation, but the results do suggest the need for further study of this phenomenon and of the sulfhemoglobinemia problem. The results, however, are quite clear in showing that diflubenzuron does not inhibit the biosynthesis of hexosamine-containing compounds such as hyaluronic acid in a manner analogous to the inhibition of chitin biosynthesis in insects.

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