# A Feeding Study with Diphenylamine in a Dairy Cow

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It was first shown by Smock (1955) that the antioxidant, diphenylamine (DPA) effectively controls apple scald, a respiratory breakdown of the fruit cells during storage. DPA is widely used for this purpose today. Bache et al. (1962) showed that 4 to 5 ppm of DPA was required on the harvested fruit to adequately control scald. Disposal of apple pomace has long been a problem since it decomposes very slowly in soil and cannot be used effectively as a mulch material. Apple pomace is a useful additive to the dairy cattle ration but was outlawed several years ago because of the presence of high concentrations of DDT and other chlorinated hydrocarbon residues.

Since chlorinated hydrocarbons have not been used in orchards for the past several years, there is currently much interest in again feeding apple pomace to cattle. Since substantial residues of DPA which remain on the harvested fruit would be present in a cows diet a feeding study was conducted to learn the fate of the compound in the bovine.

## Experimental

A Holstein cow weighing 534 kg. and with a daily milk production of 4.7 kg (3.6% butterfat) was catherized and fed pure recrystallized DPA at the 5 ppm level (based on a daily ration of 22.7 kg.) for 4 days. DPA in acetone was thoroughly mixed with the evening grain. The 5 ppm feeding level was chosen as a concentration somewhat above what one would ever expect to find in dairy cattle ration containing pomace. This is based on the fact that the permissible tolerance level of DPA in apples is 10 ppm and the concentration of DPA in pomace is typically about twice that occurring in the corresponding apples. The proportion of pomace normally used in the ration is only about 5%, however.

Morning and evening samples of the totally mixed milk were taken 1 day prior to feeding (control sample), daily throughout the feeding period and for 6 days thereafter. The morning and evening milk samples were combined each day prior to analysis. The total daily urine and manure samples were collected in specially constructed gutter trays. All samples were immediately frozen prior to analysis.



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In Vitro Studies

Rumen fluid. The stability of DPA in the presence of fresh rumen fluid was studied. One milliliter of a solution of DPA in acetone (500 μg/ml) was thoroughly mixed with 100 ml of fresh filtered rumen fluid and held at 38° C. At measured intervals 1 ml of fluid was removed and transferred to a 15 ml glass stoppered centrifuge tube containing 3 ml of methanol, 3 ml of water and 5 ml of hexane and shaken vigorously. One ml of the upper hexane layer was transferred to a 10 ml glass stoppered test tube and was brominated and analyzed for DPA by electron affinity gas chromatography using the published procedure (GUTENMANN AND LISK, 1963).

Liver. Possible metabolism of DPA was studied in the presence of the 10,000 X g supernatant fraction of fresh beef liver which contains microsomes and soluble enzymes. A portion of liver from a freshly slaughtered steer was immersed in 0.25M sucrose solution at 0° C and all further processing for enzyme preparation was conducted in the cold (0-4° C). A 20% liver homogenate in the sucrose solution was prepared using a Dounce homogenizer. The homogenate was centrifuged at 10,000 X g max for 30 min. Incubation mixtures contained 5 μg of DPA (100 ul of a 50 μg/ml solution in acetone), 25 μmol of magnesium chloride, 95 μmol of Tris buffer, pH 7.4, 20 μmol of glucose 6-phosphate, 1.5 μmol of TPN (NADP), and 1 ml of the enzyme (10,000 X g supernate) preparation in a total volume of 5 ml. Incubations were carried out in a 25-ml Erlenmeyer flask at 37° C in an atmosphere of air for 30 min. The flasks contained a borosilicate marble 0.5 in. in diameter and were mechanically shaken 100 times per minute on a reciprocating shaker during incubation. (These samples as well as the controls, which included either no enzyme or no substrate, were carried through the procedure in triplicate.) After 30 min the reactions were terminated by the addition of 3 ml of methanol and each incubation mixture was transferred to a 15 ml centrifuge tube using 2 ml of water for rinsing. Five ml of hexane was added and the mixture was shaken vigorously. One ml of the upper hexane layer was brominated and analyzed for DPA by gas chromatography as referenced above.

Extraction, isolation and analysis of DPA in milk, urine and feces.

Fifty grams of milk or urine (5 grams of feces) were placed in a 250 centrifuge bottle and 50 ml of methanol, 30 ml of hexane and 20 ml of diethyl ether were added. The bottle was stoppered, shaken and centrifuged. The upper layer was aspirated into a 250 ml separatory funnel. The aqueous mixture was partitioned twice more with 30 ml of hexane and 20 ml of ether and the upper solvent layers combined in the separatory funnel. The organic solvent mixture

was backwashed with 100 ml of water, dried with anhydrous sodium sulfate and evaporated to 10 ml. The solution was partitioned with 4 ml of concentrated hydrochloric acid and 10 ml of water. The acid and water layers were combined, diluted to 60 ml with water and extracted with 20 ml of hexane. (In the case of feces the above combined solvents were extracted with two, 4 ml portions of hydrochloric acid, the solution was diluted to 130 ml with water and then extracted with two, 10 ml portions of hexane. These combined portions of hexane were again extracted with 4 ml of hydrochloric acid, the acid diluted to 60 ml with water and this solution then extracted with 20 ml of hexane). One ml of the hexane was removed, brominated and analyzed for DPA as before.

Urine was also hydrolyzed with acid prior to extraction and analysis in an attempt to detect possible conjugates of DPA. In this procedure 5 grams of urine was digested (45° C) with 5 ml of ortho phosphoric acid for 2 hours. The solution was cooled, diluted with 10 ml each of water and methanol and extracted 4 times with 5 ml of hexane. The combined hexane layers were extracted with 4 ml of concentrated hydrochloric acid and the remaining procedure was identical to that used for the analysis of milk or urine.

## Results and Discussion

Residues of DPA were not detected in milk or urine. Small amounts of DPA were excreted in the feces. Fecal excretion of DPA amounted to about 1.4% (6.2 mg) of the total dose (0.454 gms). Table 1 lists the recoveries of DPA from samples and the estimated analytical limits of detection. DPA was stable in the presence of rumen fluid for 23 hours. About 50% of the 5 ppm of DPA disappeared during the 30-minute incubation with the liver fraction. No conjugates of intact DPA were detected in urine which had been hydrolyzed with acid. A study (Alexander et al., 1965) of the metabolism of DPA in rabbits and rats showed the presence of conjugates mainly of the 4-hydroxy and 4,4'-dihydroxydiphenylamine in their urine and in the feces of rats. It is possible that microsomal hydroxylation of DPA occurs in the bovine followed by conjugation and elimination. The gas chromatographic method used here would not have detected hydroxylated derivatives of DPA.

TABLE 1.

Recovery of DPA from Samples

| Sample | Added, ppm | Recovery, per cent | Detection Limit, ppb |
|---|---|---|---|
| milk | 0.02 | 105 | |
| | 0.1 | 93, 84, 105 | 4 |
| urine | 0.05 | 95 | 4 |
| | 0.1 | 97 | |
| urine (acid hydrolyzed) | 0.2 | 82.5 | |
| feces | 0.2 | 70, 73 | 20 |
| rumen fluid | 5 | 100 | |
| liver 10,000 xg supernate | 5 | 110 | |

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